Alternaria alternata Leaf Spot Pathogens: Genetics, Evolutionary History and Diagnostics

Andrew D. Armitage, MRes

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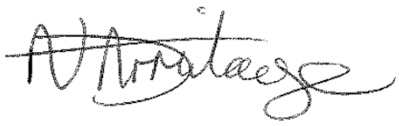
DECLARATION

I declare that the material contained in this thesis is the work of the author and has not been submitted for another degree or published previously. Content generated by collaborations, or from adapting the methodologies of previous studies are fully acknowledged in the text.

Signed:  Date: ***30/05/14***

SUMMARY

The primary objective of this study was to characterise the *Alternaria alternata* species group. Particular focus was put on understanding the pathogens *Alternaria mali* and *Alternaria gaisen*, responsible for leaf spot diseases of apple and pear and of phytosanitary importance in Europe. Understanding evolutionary relationships is important in defining the genetic and biological characteristics associated with plant pathogens in this species group. This will inform management strategies and facilitate the development of reliable detection tools for important plant diseases.

Evolutionary relationships within the *A. alternata* species group were established using a phylogenetic approach based on functional genes. Highly variable loci (*endoPG*, *Alta1*, *L152* and three novel loci) identified three major lineages within the *A. alternata* which were supported by isolate morphology. These were considered to represent subspecies within *A. alternata*.

The presence of toxin-synthesis genes, required for the production of host-selective toxins (HSTs) in apple and pear pathotypes, was established within *A. alternata* isolates. Isolates carrying apple HST-genes were only present in ssp. *tenuissima*, while isolates carrying pear HST-genes were present in ssp. *gaisen*. Virulence assays showed that apple HST-genes are required for pathogenicity on apple leaf.

The presence of different mating type genes in isolates was used to assess evidence for recombination within the *A. alternata* species group. Distribution of mating type idiomorphs indicated that recombination must have occurred in this putatively asexual species. Analysis of whole genome sequence data indicated that *A. alternata* possesses the genes required for meiotic recombination, supporting a theory of recombination and possible sexuality within this group.

This work provides insight into the evolution and causal agents of *A. alternata* plant diseases. Furthermore, whole genome sequencing data was generated during the course of this study and represents valuable genetic resource that can be used for future research, including development of pathotype-specific molecular markers.

LIST OF ABREVIATIONS

°C	Degrees centigrade
1% PDB	PDB made with 1% the recommended weight of PDB powder
AALT	*Alternaria alternata* tomato pathotype HST
ABT	*Alternaria brassicicola* HST
ACRT	*Alternaria alternata* rough lemon pathotype HST
ACTT	*Alternaria alternata* tangerine pathotype HST
AFLP	Amplified fragment length polymorphism
AFT	*Alternaria alternata* strawberry pathotype HST
AFTOL	Assembling the Fungal Tree of Life project
AKT	*Alternaria alternata* pear pathotype HST
Alta1	*Alternaria* major allergen 1
AMT	*Alternaria alternata* apple pathotype HST
ATCT	*Alternaria tenuissima* HST
ATPase	Plasma membrane ATPase
ATT	*Alternaria longipes* HST
BAC	Bacterial artificial chromosome
BLAST	Basic local alignment search tool
BLAT	BLAST-like alignment tool
bp	Base pair
BSC	Biological species concept
BSR	Biological species recognition
CDC	Conditionally dispensable chromosome
cDNA	Complementary DNA
cm	Centimeter
CO1	Cytochrome oxidase 1
CTAB	Centrimonium bromide
cv.	Cultivar
df	Degrees of freedom
DNA	Deoxyribonucleic acid
dpi	Days post inoculation
EC	Essential chromosome
EDTA	Ethylenediaminetetraacetic acid
endoPG	Endopolygalacturonase
EPPO	European Plant Protection Agency
ESC	Evolutionary species concept
ex.	Isolated / extracted from
FERA	Food and Environmental Research Agency
FUNYBASE	Fungal Phylogenomic Database
g	Gram
gDNA	Genomic DNA
GLM	General linear model
GO	Genome ontology

gpd	Glyceraldehyde-3-phosphate dehydrogenase
GSR	Genealogical concordance species recognition
het	Heterokaryon incompatibility loci
HGT	Horizontal gene transfer
HPLC	High performance liquid chromatography
hr.	Hour
HST	Host-selective toxin
IGS	Intergenic spacer
InDel	Insertion or deletion
ITS	Internal transcribed spacer
JGI	Joint Genome Institute
Kb	Kilobase
L152	*Alternaria* aegerolysin-like gene
LSD	Least significant difference
LSU	28S large ribosomal subunit
MAT	Mating type gene
Mb	Megabase
MCMC	Markov chain monte carlo
ml	Milliliter
mm	Millimeter
mRNA	Messenger RNA
MSC	Morphological species concept
MSR	Morphological species recognition
N50	The point at which 50% of contigs are present in contigs of size N or greater
ng	Nanogram
NGS	Next generation sequencing
P	Probability value
PCA	Principal components analysis
PCAgar	Potato carrot agar
PCB	Potato carrot broth
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
pM	Picomole
PSC	Phylogenetic species concept
PSR	Phylogenetic species recognition
QBOL	Quarantine pest identification database
RAPD	Random amplified polymorphic DNA
rDNA	Ribosomal DNA
RFLP	Restriction fragment polymorphism
RIP	Repeat induced point mutation
RNA	Ribonucleic acid
RNase	Ribonuclease
RNAseq	Transcriptome sequencing
RPB2	RNA polymerase subunit 2

rpm	Rotations per minute
SDW	Sterile distilled water
SE	Standard error
SNP	Single nucleotide polymorphism
sp.	Species
spp.	Species (pl.)
ssp.	Subspecies
SSU	18S nuclear ribosomal subunit
TEF	Translation elongation factor 1 alpha
TFREC	Tree Fruit Research and Extension Centre
UoW	University of Warwick
USDA	United States Department of Agriculture
V8B	V8 juice broth
µl	Microliter

CHAPTER 1

GENERAL INTRODUCTION

Emergence of new infectious plant diseases is driven by anthropogenic and environmental change including changes in trade, land use and climate (Anderson *et al.*, 2004). Movement of infected plant material through trade in plant products, germplasm, grafts and live plants has been recognised as the greatest contributing factor to the emergence of new plant diseases (Anderson *et al.*, 2004). A pathogen may be introduced without disease emergence initially until a second factor, such as introduction of disease vectors, more appropriate hosts or changes in the environment, leads to an increase in disease incidence, geographic range or severity (Anderson *et al.*, 2004). Fungi are responsible for a large number of introduced plant diseases, with more fungal diseases introduced to Europe and Africa over the 20th Century than bacteria and viruses combined (Waage *et al.*, 2008). Understanding the evolutionary history, evolutionary potential and pathogenicity of fungal diseases will aid management and identification of emerging pathogens.

Lifestyles of *Alternaria* spp.

Alternaria spp. are ubiquitous fungi. They are present in the human environment, being commonly found in environmental dust samples and air conditioning systems while spore traps often show evidence of *Alternaria* dispersal (Hjelmroos, 1993). *Alternaria* spp. have even been shown to be associated with insects, having been isolated from the backs of cockroaches (Fotedar *et al.*, 1991). Little work has been performed to investigate the saprotrophic lifestyle of *Alternaria* spp., which probably accounts for the majority of *Alternaria* species in nature (Thomma, 2003). *Alternaria* spp. are capable of persisting on low nutrient media, suggesting that they can complete their lifecycle in poor nutrient environments.

Alternaria are best known for their role as plant pathogens and the USDA Fungal Host Index contains over 4,000 plant-host associations in this genus, ranking it 10th in terms of total number of host associations out of nearly 2000 fungal genera (Bills *et al.*, 1987). The *A. alternata* species group alone is recorded as causing disease on over 100 host plants (Thomma, 2003). This includes economically important crops including cereals, ornamentals, vegetables and fruits, with losses incurred through direct crop damage, postharvest spoilage or through contamination with mycotoxins.

Alternaria infections usually occur on leaves and stems of the host plant (Fig. 1.1: *a*). Leaf spots are recognised by black necrotic lesions surrounded by chlorotic halos. Leaf necrosis may lead to reduced marketability for leafy crops such as *Brassica*, and may also result in the host abscising leaves, hence reducing photosynthetic potential and crop yields indirectly, as is the case in apple and pear. Fruit spotting is also caused by *Alternaria* spp. leading to reduced crop marketability, a significant problem in citrus fruits.

Economic losses are also incurred by *Alternaria* spp. post harvest. In Red Delicious varieties of apple in South Africa, annual losses of 6-8% have been attributed to *Alternaria* dry core rot (Combrink *et al.*, 1985). Such post harvest diseases are often not thought to be attributed to a single *Alternaria* sp., but may be caused by a range of species (Serdani *et al.*, 2002). Infections of apple fruits by *Alternaria* spp. occur in the field and in storage, where they are favoured by low temperatures. This reflects the saprotrophic / opportunistic necrotrophic lifestyle common through *Alternaria* species.

Post-harvest spoilage may not just be a result of visual blemishes or reduced palatability but may also be caused by mycotoxin contamination. Mycotoxins are non-host selective toxins produced by fungi and more than 30 have been isolated from *Alternaria* (Robiglio and Lopez, 1995). Toxins are produced by *Alternaria* infecting crushed fruit, as demonstrated on crushed apples, tomatoes and blueberries (Stinson *et al.*, 1980), but also on intact produce, such as on whole tomatoes, apples oranges and lemons (Stinson *et al.*, 1981). These have been shown to pose a range of human health risks (Brugger *et al.*, 2006, Lehmann *et al.*, 2006) including being linked to human oesophageal cancer (Liu *et al.*, 1992). *Alternaria* mycotoxins are frequently detected in fresh produce including fruit products and juices, as well as grains such as wheat and plant oils (Torres *et al.*, 1993, Ostry, 2008, Muller and Korn, 2013). The species responsible for contamination are often reported to be *A. infectoria* or *A. alternata* (Muller and Korn, 2013).

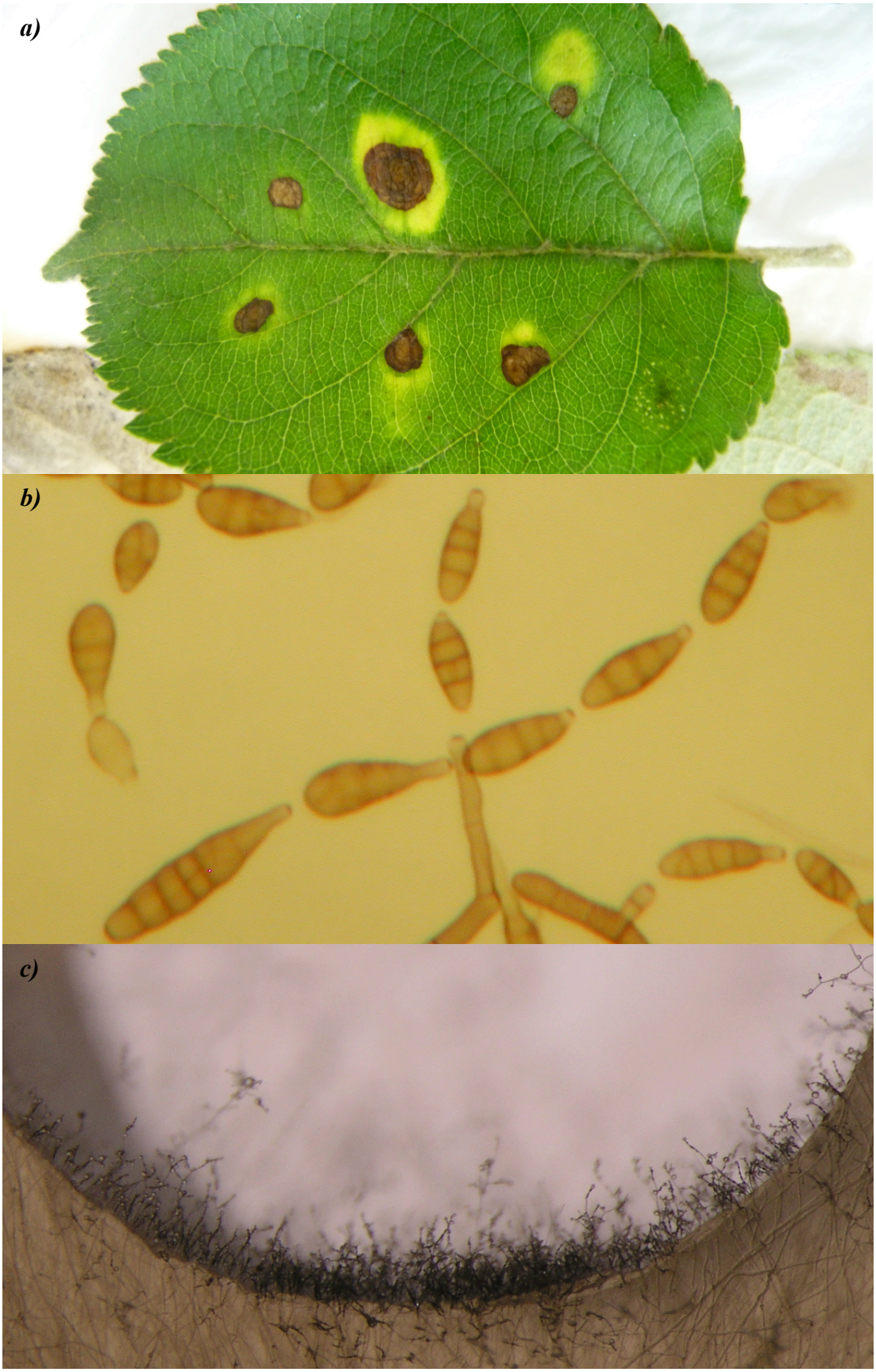


Figure 1.1 Images of the *Alternaria alternata* apple pathotype: a) Causing disease on apple leaf, b) spores at 200x magnification showing characteristic lateral and transverse septation and c) chains of spores produced from conidiophores growing out of agar medium.

The *Alternaria* genus, and particularly the species *A. alternata*, are also of clinical significance often associated with human airway disorders including allergy, asthma and chronic rhinosinusitis (Hopkins *et al.*, 1930, Achatz *et al.*, 1995, Callejas and Douglas, 2013). As a result, *Alternaria* spp. are considered to have a large contribution to the 3 billion US dollars spent on the relief of allergenic rhinitis each year in the USA (Bush and Prochnau, 2004). *Alternaria* spp. are also gaining recognition as human invasive pathogens. This usually occurs in immuno-compromised patients (Vartivarian *et al.*, 1993), occurring as lung or sub-cutinal infections (Marcoux *et al.*, 2009, Schultze-Werninghaus, 2012). Infection also occurs following surgery requiring antifungal treatments or further operations to remove infection (Boyce *et al.*, 2010).

Characterisation of *Alternaria* spp.

First description of the genus

The genus *Alternaria* was first described in 1817, with *Alternaria tenuis* as the type isolate. Keissler (1912) found ambiguities in descriptions of *A. tenuis* and synonymised both *A. tenuis* and *Torula alternata* to *Alternaria alternata*. No sexual stage was evident in the genus and as such it was classified in the Phylum Fungi Imperfecti with other asexual fungi. Since the genus' conception, over 1000 *Alternaria* species have been described. Many of these species names are invalid as they have since been classified into other genera, or because they lack type specimens (Simmons, 2007). The continued revision of the genus reflects its diverse nature, possessing considerable variation in spore structure and being identified in many different ecological niches.

Morphological descriptions

Most classification of the *Alternaria* spp. has been based on morphology. This understanding was brought together through the life-work of E.G. Simmons who published 355 essays and papers on *Alternaria* morphology (Simmons, 1967,

Simmons, 1981, Simmons, 2003). This work was subsequently summarised in an identification guide for the *Alternaria* genus, re-describing 275 morphological species (Simmons, 2007).

The *Alternaria* genus is characterised by large, multicellular, melanised conidia (Fig. 1.1: *b*), which can possess both longitudinal and transverse septae. Spores are typically broadest at the base and taper towards the end. The tapering at the end of spores is commonly referred to as a "beak". Spores are produced on conidiophores often in chains, that may be branching or lead to secondary conidiophores that produce further spores. It is mainly the individual spore characters and the patterns of sporulation that are used to differentiate morphological species within the genus (Fig. 1.1: *c*).

Identification of *Alternaria* taxa has long been considered problematic. Over 1000 species have been described and 275 names are in current use. Frequent revision of groups in the genus has resulted in the species boundaries being unclear. Taxonomic keys based on morphology have been attempted but have not contained appropriate characters to identify taxa at what has been commonly considered a species level (Simmons, 1992, Simmons, 2007). Overlapping spore characters and natural variation in response to culturing conditions made these keys hard to follow, particularly to users operating outside of the author's own lab. This was particularly true for many small-spored *Alternaria* spp. (including *A. alternata*), which display considerable morphological diversity, are present ubiquitously in the environment and exhibit adaptation to a variety of lifestyles; from economically important plant pathogens to human allergens. Therefore, broader groups of spore morphologies were developed to categorise these species (Simmons and Roberts 1993) and are described in detail in Chapter 4. This "lumping" of morphologically described species did much to simplify identification of *Alternaria* spp.. Whether these morphological groups each represent multiple distinct species or actually represent a smaller number of highly variable species is still unresolved.

Toxin characterisations

Concurrent to major revisions of taxa on the basis of *Alternaria* morphology (Simmons, 1981, Simmons, 2003), mycotoxins were being identified and characterised in *Alternaria* species. Toxins that were associated with plant disease on major fruit crops were of particular interest. Morphologically similar *A. alternata* species were found to produce toxins that conferred "host-specific" pathogenicity on apple, pear, tomato, strawberry, tangerine or rough lemon plants (Nishimura *et al.*, 1974, Okuno *et al.*, 1974, Gilchrist and Grogan, 1976, Maekawa *et al.*, 1984, Kohmoto *et al.*, 1993). Later it was shown that these toxins had a broader host range than originally thought leading to them being referred to as host-selective toxins (HSTs) (Itoh *et al.*, 1993). The discovery and characterisation of these toxins is discussed in detail in Chapter 5.

Conflict between *Alternaria* morphological species descriptions (Simmons, 1999b), and results from newly introduced molecular techniques (Kusaba and Tsuge, 1995b) have resulted in ambiguity over which morphological descriptions constitute species. Multiple morphological species descriptions are available for HST producing *Alternaria*, but all of these taxa possess identical DNA sequences for the internal transcribed spacer region (ITS) and as such have been considered as a single species, *A. alternata* (Kusaba and Tsuge, 1995b).

As a result of differences of opinion in naming the HST-producers, some *Alternaria* pathotypes have both morphological species descriptions and pathotype designations. This has led to confusion when naming the agents of a disease, for example: *A. mali* was described as the causal agent of an infection of apple trees in the USA and the description of this species was based on spore morphology. Separate from the morphological description of *A. mali* is its pathotype designation. Individuals that are capable of producing apple HSTs are termed *Alternaria alternata* apple pathotypes, and were first identified in Japan. In general, current literature describes HST producing individuals as pathotypes of their particular host (e.g. *Alternaria alternata* apple pathotype is the cause of Alternaria leaf blotch), but often scientific literature and disease regulation use the two names interchangeably (e.g. *A. alternata* tomato pathotype referred to as *A. arborescens* in Hu *et al.* (2012)), despite the names

representing two different species concepts and there being little evidence that morphological species even cause the same disease.

DNA based approaches

The development of molecular approaches has advanced our understanding of evolutionary relationships in *Alternaria* genus. Many morphological described species have been confirmed as distinct evolutionary lineages including *A. brassicicola*, *A. infectoria*, *A. porri* and *A. radicina* (Kusaba and Tsuge, 1995b, Cooke *et al.*, 1998, Pryor and Gilbertson, 2000, Lawrence *et al.*, 2013, Woudenberg *et al.*, 2013). However, in many cases, multiple morphological species are associated with a single phylogenetic lineage. These lineages generally reflect taxa that have previously been considered to be morphological species groups and have recently been described using the taxonomic level section (Fig.1.2, Lawrence *et al.* (2013), and subsequently in Woudenberg *et al.* (2013)). The *Alternaria* section *Alternaria* relates to what was previously considered the "*Alternaria alternata* species group". The taxonomic status of this group is still unresolved, as molecular approaches have shown limited resolution between morphological species (Andrew *et al.*, 2009). Individuals within this group are generally considered to represent a single species *A. alternata*. Accurate classification of this group is required due to its diversity of roles as an environmental saprophyte, human allergen / pathogen, and plant pathogen.

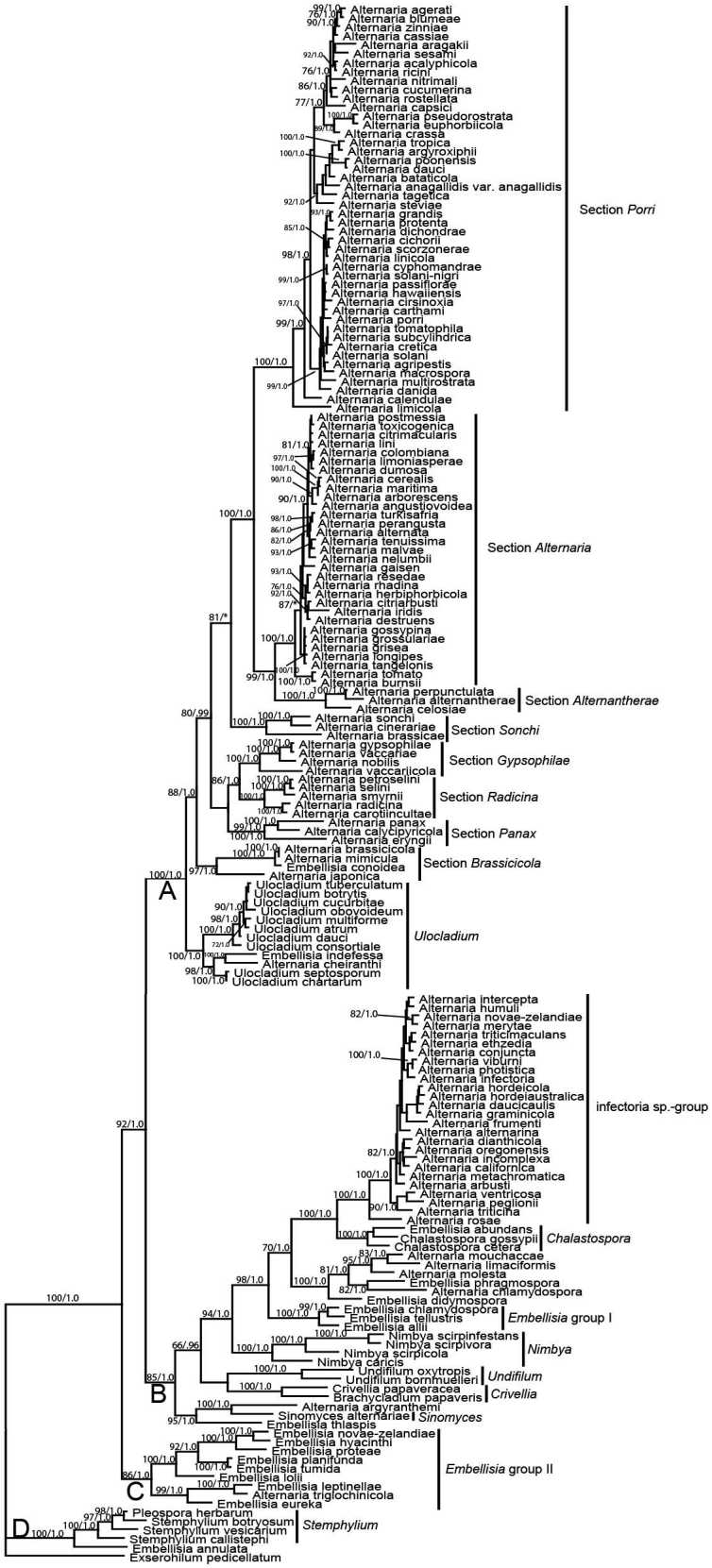


Figure 1.2 Classification of the *Alternaria* genus as presented in Lawrence *et al.* (2013): Phylogeny based upon five highly variable loci, as discussed in Chapter 3. Four major lineages are identified within the *Alternaria* genus (*A*-*D*). Morphologically similar species groups are named as sections. The *A. alternata* species group is named *Alternaria* section *alternaria*. Image adapted from (Lawrence *et al.*, 2013).

Spread of non-native *Alternaria* spp.

Alternaria pathogens of pome fruits cause significant economic damage in their native ranges. The *A. alternata* apple pathotype is one of the most important pathogens of apple in Japan and other Asian countries (Filajdic *et al.*, 1995). For example, in China Alternaria blotch is regarded as one of the four most serious diseases of apple in all major apple-producing regions (Li *et al.*, 2013). Fungicide control is frequently used (Smith *et al.*, 1996) but this has an impact in terms of cost and the environment. In apple, up to 85% of leaves can be infected in susceptible plants whereas in resistant cultivars, only 1% of leaves are infected (Abe *et al.*, 2010). The market value of crops can be further reduced through blemishes (spots) on the fruits of susceptible cultivars. *Alternaria* spp. are also responsible for post-harvest diseases of fruit. In *Malus* this is known as dry core rot. Susceptible cultivars are those with open calyx such as red delicious varieties (DEFRA, 2004). The *A. alternata* pear pathotype (syn. *A. gaisen*) causes significant economic loss through leaf spots and fruit-spots on Asian pear (*Pyrus pyrifolia*) in East Asia. The two pathotypes specific to pome fruits have been shown capable of establishing outside of their native geographic ranges.

Spread of Alternaria leaf blotch has been linked to increased planting of susceptible apple cultivars (Li *et al.*, 2013). *Alternaria* diseases of apple and pear are generally of minor importance in Europe. This may be due to resistance already being present in commonly grown varieties. However, the *A. alternata* pear pathotype has been detected in Asian pear (*Pyrus pyrifolia*) orchards in Southern Europe (Fig. 1.3: *a*) and the apple pathotype *A. mali*, has recently been reported as a significant disease in Italy (Rotondo *et al.*, 2012) (Fig. 1.3: *b*).

The UK Food and Environmental Research Agency (FERA) is obliged to "take action" on (including rejection of) shipments of material identified as carrying pathogenic non-native small-spored *Alternaria*. This leads to economic loss to producers and distributors. This was highlighted in December 2003 when a novel morphological species of *Alternaria* (*Alternaria yaliinficiens*) was described on Ya Li pears imported from China to the USA; as a result over three million pounds worth of Chinese pears were removed from supermarket shelves and further imports were restricted until 2006 when American phytosanitary bodies were satisfied with control

methods implemented by Chinese growers (R.G. Roberts, personal communication). To ensure control is implemented properly by phytosanitary bodies and by growers, effective and accurate diagnostics are required for *Alternaria* pathogens.

Host ranges of individual pathotypes within *A. alternata* are not yet understood, for example, pathotypes of *A. alternata* thought to be specific to lettuce, tomato and strawberry have each been shown to be capable of causing leaf lesions on European pear (*Pyrus communis*) (DEFRA, 2004). Furthermore, there is evidence that some European *Malus* and *Pyrus* cultivars may be less resistant to *Alternaria* diseases than cultivars grown inside the disease's natural host range (DEFRA, 2004). The European and Mediterranean Plant Protection Organisation (EPPO) lists *A. gaisen* as a documented pest and it lists *A. mali* as an A1 quarantine pest; meaning that it is not present and is recommended for regulation throughout the EPPO region.

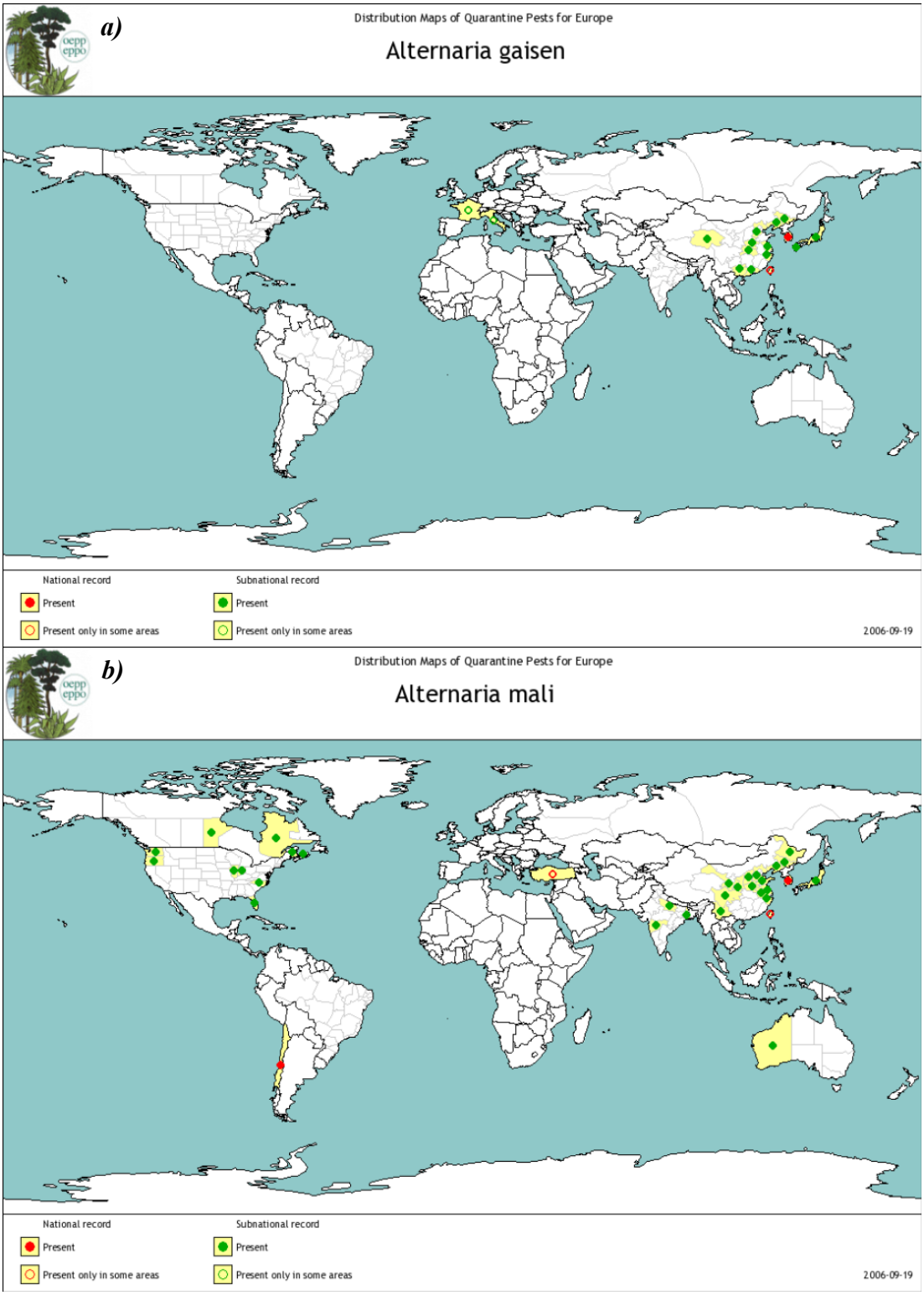


Figure 1.3 Distribution maps of quarantine pathogens *Alternaria gaisen* and *Alternaria mali*: Showing registered identifications of *a*) *Alternaria* causing disease on pear leaves (*A. gaisen* or the *A. alternata* pear pathotype), and *b*) *Alternaria* causing disease on apple leaves (*A. mali* or the *A. alternata* apple pathotype). Native ranges are considered to be in East Asia (both pathogens) and the Americas (apple pathogen). Image taken from EPPO (2006).

Molecular ID of *Alternaria* taxa

Identification of quarantine material

The accuracy and effectiveness of phytosanitary regulatory activities are dependent upon correct identification of quarantine material (Roberts *et al.*, 2011), which in turn is dependant upon an accurate taxonomic framework (Narayanasamy, 2011). Conventional methods of disease diagnosis involve the study of symptoms on the host, isolation of fungi in suitable culture media and determining the characteristics of sexual and asexual structures along with spores which are used for the taxonomic identification of fungi (Narayanasamy, 2011). Isolates need to be assigned a taxonomic name as a process of registering and monitoring the occurrence of disease. In the *Alternaria* this has traditionally relied upon observations of sporulation structures under standard conditions (Simmons, 2007). Identification based upon molecular methods, rather than morphological examination, is becoming more prevalent in modern diagnostic laboratories. Molecular methods offer generic tests, meaning that less expertise needs to be maintained allowing delivery of a cost-effective sustainable service (Boonham *et al.*, 2008). Molecular methods can also offer advantages in speed (as a culturing step is not always required), automation, can be performed at a high-throughput, and are less ambiguous in their results. Genetic markers, showing resolution between quarantine and non-quarantine pathogens are therefore required (Seifert, 2009).

Recent progress has been made in resolving the taxonomy of the *Alternaria* genus and related organisms (Lawrence *et al.*, 2013, Woudenberg *et al.*, 2013) but the *A. alternata* species group (syn. *A.* Section a*lternaria*) is still unresolved. The quarantine pests *A. mali* and *A. gaisen* belong to this unresolved group. Current barcoding loci need to be assessed for their suitability to differentiate quarantine from non-quarantine species within the *A. alternata* species group. Where commonly used barcoding loci do not differentiate taxa, then more specific barcoding loci must be developed for specific groups (Seifert, 2009). Variable phylogenetic loci that show species and population level variation in other systems need to be assessed for their potential as secondary molecular markers across *Alternaria* taxa. If previously used

loci do not show resolution then novel loci may be developed, taking advantage of the increased access to genomic sequence data.

Barcoding loci

DNA barcoding is an approach to rapidly identify species using short, standard genetic markers (Dentinger *et al.*, 2011). Barcoding uses a set of primers with broad specificity to amplify genetic regions that are typically 500-800 bp in length (Schoch *et al.*, 2012). Cytochrome oxidase subunit 1 (CO1) is the animal barcoding locus and as such has been commonly used in fungi. The cytochrome oxidase locus has been concluded to be less appropriate for barcoding of fungi than the internal transcribed spacer (ITS) region (Dentinger *et al.*, 2011). The ITS locus is a region of ribosomal DNA (rDNA) cistron covering two noncoding regions between the 18S and 28S rDNA and including the 5.8S rDNA (Fig. 1.4). The structure of the locus is conserved enough across the fungal kingdom to allow for high amplification success using standard primers (White *et al.*, 1990), and shows high levels of inter-specific variation and intra-specific variation (Schoch *et al.*, 2012). It is widely considered as the fungal barcoding region (Schoch and Seifert, 2012). This is reflected in the prevalence of sequencing of this locus in routine diagnostics, as of February 2012 there were over 172,000 full-length fungal ITS sequences deposited on Genbank, representing 15,000 species (Schoch *et al.*, 2012).

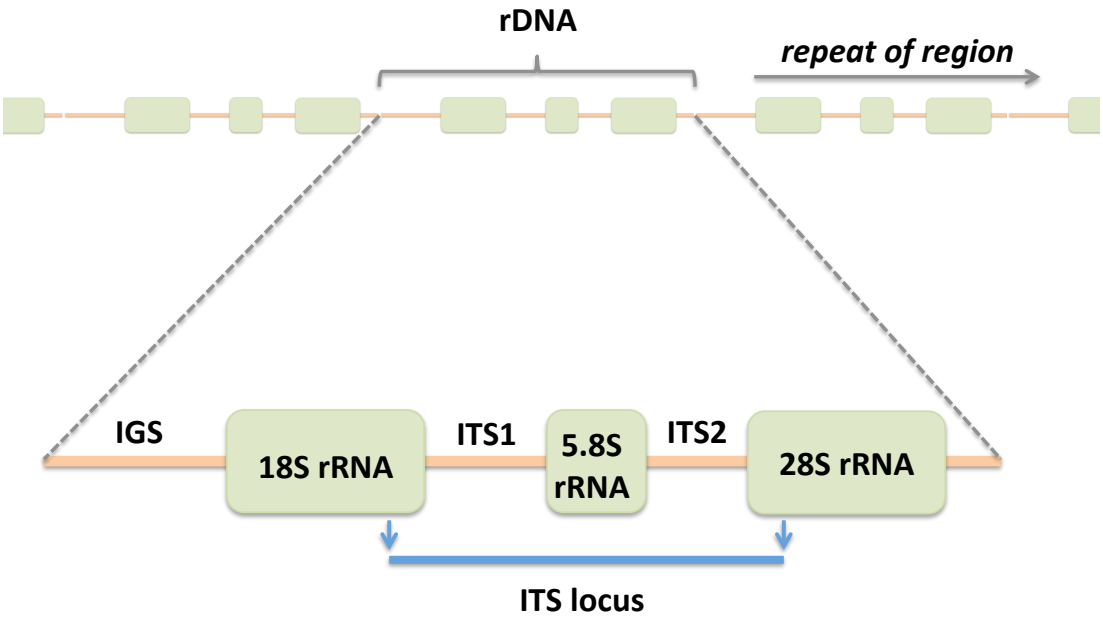


Fig 1.4 Structure of fungal nuclear ribosomal DNA: Showing regions coding for 18S rRNA, 5.8S rRNA and 28s rRNA. Non-coding sequences at the inter-genic spacer (IGS) and internal transcribed spacer regions (ITS1 and ITS2) are shown. The position of the "ITS locus", a region commonly used for barcoding is marked.

The ITS region has been reported as showing no inter-specific variability within some species-rich fungal genera such as *Penicillium* and *Fusarium* (O' Donnell and Cigelnik, 1997, Skouboe *et al.*, 1999) which has led to criticism in its use as a barcoding gene (Kiss, 2012). It has shown some resolution within the *Alternaria* genus, being used to identify major morphological species groups and differentiate the *A. alternata* species group from other small-spored species such as *A. infectoria*. The locus has shown little or no resolution when studies have attempted to resolve morphologically described species within the *A. alternata* species group (Kusaba and Tsuge, 1995a, Pryor and Gilbertson, 2000, Cho *et al.*, 2001, Serdani *et al.*, 2002, Lawrence *et al.*, 2013, Woudenberg *et al.*, 2013). This had led to the suggestion that morphological species described within the *A. alternata* species group can be considered as intraspecific variants of *A. alternata* (Kusaba and Tsuge, 1995a).

Other commonly used barcoding loci have been used to investigate phylogenetics within the *Alternaria* genus. The 18S nuclear ribosomal subunit rDNA (SSU) and 28S large ribosomal subunit (LSU; Fig. 1.4) are commonly used loci in phylogenetics of fungi and have been evaluated as potential fungal barcoding loci (Schoch *et al.*,

2012). Throughout the fungal kingdom the SSU has shown greater levels of intraspecific variation than the ITS region, and the LSU region is recognised as showing a higher resolution than the ITS region in the Ascomycete yeasts but is inferior to ITS in other regions of the fungal kingdom (Schoch *et al.*, 2012). Both loci have been used to resolve the *Alternaria* genus (Woudenberg *et al.*, 2013) but have previously been reported to show low variability within the *alternata* species group (Pryor and Gilbertson, 2000, Peever *et al.*, 2004). Where standard barcoding loci do not show resolution within taxa, then other loci showing greater variability are required. These are often based upon single copy genes rather than on rDNA regions. These are discussed in detail in Chapter 3.

Species concepts and species recognition

When dealing with a taxonomically difficult group such as the *Alternaria* genus clear concepts are needed for what constitutes a species. Our understanding of what defines species are based upon theoretical frameworks known as species concepts. Species concepts can include any framework to understand what a species is and may be based on theoretical frameworks from divine creation to Darwinian evolution. Mayden (1997) identified 22 species concepts currently in use. The most notable of these is the evolutionary species concept (ESC) that defines a species as *'a single lineage of ancestor-descendant populations which maintains its identity from other such lineages and which has its own evolutionary tendencies and historical fate'* (Wiley, 1978). Many species concepts, such as the ESC, are purely theoretical and cannot necessarily be used to diagnose or recognise species (Mayden, 1997). Some species concepts are less theoretical and can be considered as operational species concepts. These include morphological, biological and phylogenetic species concepts. These could be considered inferior to the ESC at defining the concept of species but are superior in their application to biological problems. Tests based upon operational species concepts can be termed "species recognition" criteria (Taylor *et al.*, 2000). The ESC is generally accepted as explaining a species, however it is not very useful for species recognition. Other species concepts as described below are more useful:

Morphological species concept

The morphological species concept (MSC) has been the primary approach to delimit fungal species. It has been responsible for the description of most of the 70,000 fungal species described to date (Taylor *et al.*, 2000). Morphological species recognition (MSR) uses phenotypic traits to deliminate species. A definition by Cronquist (1978) states that '*species are the smallest groups that are consistently and persistently distinct and distinguishable by ordinary means*'. It has been applied in the *Alternaria* genus, using characters such as spore morphology, growth on different media and production of different metabolites to identify species (Simmons, 1981, Simmons, 2003, Andersen *et al.*, 2009). The MSC recognises discrete phenotypic units but may not necessarily reflect the ESC. It has little consideration for an organism's evolutionary tendencies and struggles with subspecific variation such as local adaptation. The *Alternaria* genus is primarily comprised species described by E.G. Simmons. His approach to describing species reflected the subjective nature of species concepts: *'If the taxon under observation has stable colony development in anexic culture, distinctive sporulation features, and microscopic characters distinguishable from those of similar taxa, then the taxon requires a unique tag to hold its place for retrieval in the published literature'* (Simmons, 2007). This can be summarised as considering a species to be any discrete taxonomic unit.

Biological species concept

The biological species concept (BSC) is commonly used in sexual fungi. It was described by Mayr (1940) as *'groups of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups'*. This speciation concept considers limits of gene flow between populations to be species boundaries, meaning that species defined under this concept will have distinct evolutionary tendencies and historical fate. Furthermore it is a testable species concept as biological species recognition (BSR) can use mating behaviour and ability to produce viable offspring to test the presence of species boundaries. Problems arise in many fungal groups, as approximately 20% of fungi are considered asexual

(Reynolds, 1993). In these clonal species, an absence of any genetic exchange requires the concepts used recognise species to be considered (see "speciation mechanisms" below). Evidence of sexuality is being demonstrated in an increasing number of fungi (Dyer and O'Gorman, 2012), but may still be cryptic and therefore not of practical use in deliminating species boundaries. This is the case within the *Alternaria* genus. The genus was originally placed in the Fungi Imperfecti, due to a lack of a sexual stage and although early there is a some evidence for sexuality in the *Alternaria* genus (Chapter 6), morphological structures associated with sexuality have not been observed in the majority of the *Alternaria* spp. (Perello and Sisterna, 2008). Therefore BSR has not been applied to this group.

Phylogenetic species concept and genealogical concordance

Phylogenetic species concepts (PSC) consider distinct clades in genetic phylogenies to represent species. Phylogenetic species recognition uses the framework of Cracraft (1983) where *'the smallest diagnosable cluster of individual organisms within which there is a pattern of ancestry and descent'* delimits a species. Similar to the morphological species concept this definition struggles to acknowledge the possibility of subspecies or population level variation. As such, PSR often uses other species recognition concepts to support it (Baum and Donoghue, 1995). A development of the PSC was incorporation of principles from the BSC. This led to genealogical concordance species recognition (GSR) (Avise and Ball, 1990). This denotes species boundaries by identifying limits of gene flow between populations as indicated by incongruity between multiple genetic phylogenies. Where tree topologies are identical then there is no gene flow between populations as polymorphic sites have become genetically fixed (due to selection or genetic drift). If gene flow can occur between populations then populations do not have their own "historical fate" and do not have distinct "evolutionary tendencies" from each other. Where gene flow does not occur then we can consider these groups as distinct species (Fig. 1.5).

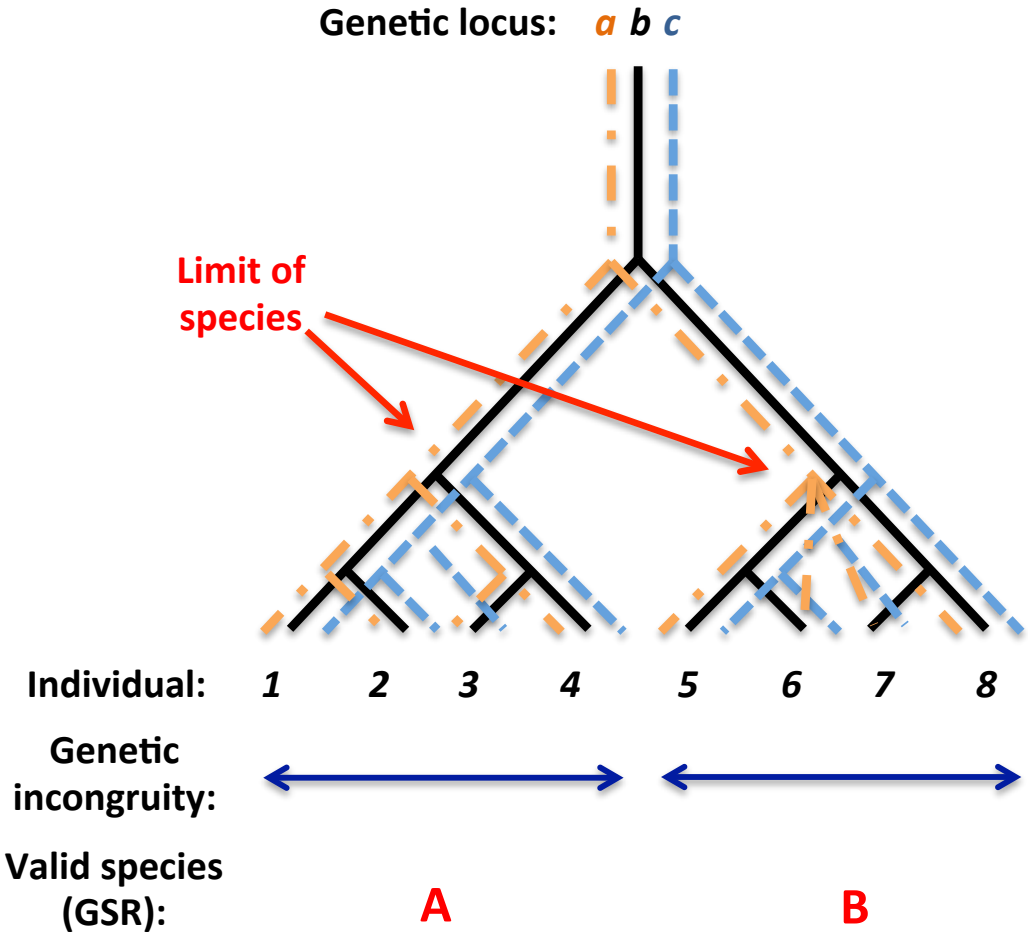


Figure 1.5 Explanation of genealogical concordance species recognition: Gene flow is present where there is incongruity between tree topologies (as shown in genetic loci a, b and c). In this example evidence of gene flow is shown within two groups of individuals but not between them. These groups can be considered species.

Similar to the BSR, GSR cannot be clearly applied to asexual fungi. This stems from their basis on the ESC, which identifies a species upon the basis of strains being a single lineage, having their own evolutionary tendencies and historical fate and following this criteria each progeny from an asexual species could be considered a new species. To deal with this problem GSR denotes the species boundary to be the point at which sexuality was lost and recombination cannot be detected (Taylor *et al.*, 1999) (Fig. 1.6). Application of this form of GSR would be expected to lump whole genera of asexual species into single species. This approach has not been widely tested but evidence of cryptic sexuality and parasexual recombination is showing greater gene flow in putatively asexual species than previously thought (Dyer and O'Gorman, 2012, Stewart *et al.*, 2013).

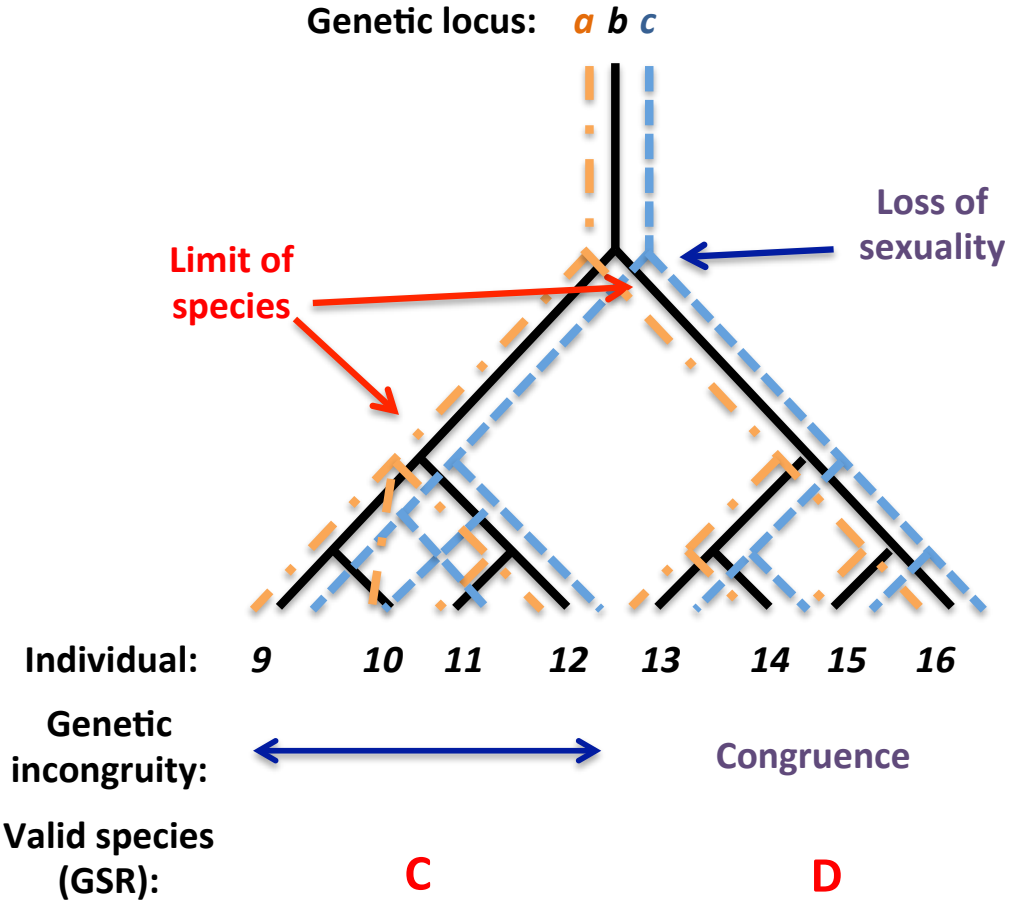


Figure 1.6 Explanation of genealogical concordance species recognition in clonal species: In this example evidence of gene flow (incongruity between loci a, b and c) is shown within one group of individuals, but loss of sexuality (and recombination) means that the second group are clonal. The clonal population should be considered species, with a boundary at the loss of sexuality.

Often combinations of species recognition criteria are employed. Distinct groups of genetically isolated individuals identified by GSR and supported by MSR may be the closest that species can get to satisfying the definition of the ESC. Where species are truly asexual (and do not undergo recombination) then a combination of the MSR and PSR may be considered the closest we can satisfy an ESC.

Speciation mechanisms

Speciation is the process of genetic isolation and divergence of a population from its parental population in accordance with the ESC. A number of events can lead to speciation. The following have been suggested as mechanisms in fungi (Giraud *et al.*, 2008):

Allopatric speciation - A geographic barrier prevents gene flow between two previously connected populations. Genetic divergence occurs between the two populations leading to phenotypic divergence and formation of reproductive isolating mechanisms. Variations of the allopatric speciation concept involve peripatric speciation (geographical isolation of a population, followed by its adaptation to a new niche) and parapatric speciation (incomplete geographical isolation of one population which then adapts to a new niche).

Allopatric speciation is an intuitive speciation concept as extrinsic geographic barriers are clear impediments to gene flow (Giraud *et al.*, 2008). Some fungi show potential for long-distance dispersal of spores through air currents and clouds, questioning the extent to which total genetic isolation occurs (Finlay, 2002, Amato *et al.*, 2007). However examples of allopatric speciation have been identified. For example *Saccharomyces paradoxus*, present in temperate woodlands in the Northern hemisphere. Eurasian populations have been shown to form distinct genetic groups from North American populations, indicating divergence of populations between continents (Kuehne *et al.*, 2007). Allopatric speciation has been shown to be present in plant pathogens. Multi-locus sequencing and GSR by O'Donnell *et al.* (2004) was used to describe nine species in *Fusarium* of which four species (*F. cortaderiae*, *F. brasiliucum*, *F. austroamericanum* and *F. meridionale*) were shown to be restricted to a geographical distribution of South America. Two further species, *F. mesoamericanum* and *F. asiaticum*, were only found in Central America and Asia respectively (O'Donnell *et al.*, 2004).

Sympatric speciation - Speciation is independent of geography. Host switching can be considered as an example of sympatric speciation, and may be a mechanism of speciation in plant-pathogenic fungi. In populations where host switching has occurred then different individuals may show adaptations to one of the two separate

hosts. One method is that recombination between two individuals that each possess specialised adaptation to one of two hosts may produce progeny that have reduced fitness when infecting either of the two hosts. This leads to a reduction in gene flow between the two populations leading to further divergence. This can be summarised by considering sympatric speciation as speciation by specialisation. Evidence of sympatric speciation has been provided by Peever (2007) who performed multi-locus sequencing on a worldwide sample of *Ascochyta* fungi causing disease on chickpea, broad bean, lentil and pea. Evidence was provided for strong host specificity of fungi to each host, yet crosses between host specific lineages revealed that lineages were completely inter-fertile (Peever, 2007). This showed that adaption to a particular host was enough to act as a barrier to recombination between species (Peever, 2007).

Speciation by hybridisation (alloploidy) - Two divergent fungal populations may not be completely intersterile and formation of heterokaryons may still occur between them. Partial sterility may lead to the formation of hybrids between two divergent fungi. Hybridisation events may result in fungi that have the same number of chromosomes as their parents and are termed allodiploid or allohaploid. Or they may have chromosomes equal to the total number of chromosomes of the two parents and are termed allopolyploid. Hybrids may have selective advantages over parental species due to them being able to exhibit wider ranges of phenotypes allowing them to exploit new ecological niches. Progeny may be sterile due to allopolyploids having a number of chromosomes incompatible with either of the parental populations. These may be considered new species. Speciation by hybridisation has been proposed to be the origin of the *Verticillium longisporum* pathogen, from *V. dahliae* and a second (unknown) parent related to *V. albo-atrum* (Inderbitzin *et al.*, 2012). Hybridisation led to an increase in host range, establishing *V. longisporum* as a major disease of oilseed rape (Inderbitzin *et al.*, 2012). *V. longisporum* isolates are stable diploids, with alleles from both parents present in the genome (Inderbitzin *et al.*, 2012). Multi-locus sequencing has shown that *V. longisporum* must have arisen at least three times in independent hybridisation events (Inderbitzin *et al.*, 2012).

Loss of sexuality - The use of species concepts has a large influence on how we think of speciation in the 20% of fungi that are asexual (Reynolds, 1993). When recombination does not occur between lineages then one may interpret speciation in a number of ways. Firstly, each individual is genetically isolated and is a single lineage

and therefore constitutes a species; secondly, populations of clonal individuals are subject to the same selection pressures and therefore the population has a common evolutionary history and potential future. GSR considers that a loss of recombination limits the application of species concepts. As discussed above, it states that we treat a species as the group of lineages linked at the point of which genetic exchange was lost.

Recent developments in whole genome sequencing

In 1977 the 5,376 bp of bacteriophage φX174 was the first genome to be sequenced (Sanger *et al.*, 1977a). This made use of recently developed "Sanger sequencing" by PCR termination with dideoxynuceotides and visualisation on agarose gels (Sanger *et al.*, 1977b). Development of capillary sequencers in the 1990s removed the need for agarose gels allowing "high-throughput sequencing", leading to sequencing of the first eukaryotic genome *Saccharomyces cerevisiae* (Goffeau *et al.*, 1996) and other fungal genomes such as *Aspergillus fumigatus* (Nierman *et al.*, 2005) and *Magnaporthe grisea* (Dean *et al.*, 2005). Sanger sequencing was also used to sequence genomes of a number of important Dothideomycete plant pathogens such as *A. brassicicola*, *Cochliobolus heterostrophus*, *Mycosphaerella graminicola*, *Phaeosphaeria nodorum* and *Leptosphaeria maculans* (Hane *et al.*, 2007, Goodwin *et al.*, 2011, Hane *et al.*, 2011, Rouxel *et al.*, 2011). Next generation sequencing (NGS) machines used "sequencing by synthesis" approaches to remove the electrophoresis step altogether, increasing throughput and further reducing the cost of sequencing (Hane *et al.*, 2011). This has led to widespread sequencing and re-sequencing of fungal genomes; reflected by the recent publication of 14 novel Dothideomycete genomes (Ohm *et al.*, 2012).

NGS machines are continually improving in read-length, throughput and cost per base. Furthermore NGS technology is available to smaller research groups through development of benchtop sequencers such as the MiSeq (Illumina), able to generate large amounts of data (up to 8 Gb) over longer read lengths (up to 250 bp) at a low cost per run. Genome sequencing is a four-step process and after generating sequence

data (1), genomes must be assembled (2), genes predicted (3) and functional annotations given to genes (4).

Previously sequenced genomes can be used as templates for the assembly of new genomes ("referenced based assembly"). However, new genomes may contain novel regions not present in the reference genome and for this reason *de novo* genome assembly is often more appropriate. NGS technologies have posed challenges to traditional methods for the *de novo* genome assembly tools designed for Sanger sequencing (Lin *et al.*, 2011) and new programs have been developed for assembly of NGS reads. These can use a number of approaches, including being based upon the principals of de Bruijn graphs such as Velvet (Zerbino and Birney, 2008, Compeau *et al.*, 2011). Quality of genome assembly is influenced by read length, genome coverage and error rate and as such the best program is dependent on the dataset, but Velvet has generally performed well when using data from Solexa/Illumina sequencing (Feldmeyer *et al.*, 2011, Lin *et al.*, 2011, Zhang *et al.*, 2011). Once genome assembly is complete the repetitive regions in the genome (transposons, inserted viruses etc.) are usually "masked" from the assembly, replacing these regions with "N's". Quality of genome assembly is assessed by the number of contigs assembled, length of the largest contig, total size of the assembled genome and the N50; being the point at which 50% of the genome is contained in contigs of size *N* or greater (Feldmeyer *et al.*, 2011, Lin *et al.*, 2011, Zhang *et al.*, 2011). N50 values should be larger than the median gene size before gene prediction is performed (Yandell and Ence, 2012).

Following assembly, *ab initio* gene prediction is performed. In a review on gene prediction Picardi and Pesole (2010) defined a gene as *'transcribed DNA region including exons and introns regulated by cis*-acting *elements such as promoters located upstream of the gene and other regulatory elements (e.g. enhancers) located also very far away from the transcription start site. Furthermore specific sequences recognised by the splicing machinery are generally found between introns and exons, and inside introns.'* Gene prediction typically focuses on using these features, particularly start and stop codons, splice sites, polyadenylation sites, branch sites and coding sequence to identify the presence and structure of genes (Picardi and Pesole, 2010). Accuracy of gene prediction is improved through "training" the program to the organism of interest, which involves altering parameters of the program. In addition

to training, programs such as Augustus (Stanke and Waack, 2003) can incorporate aligned sequences from transcriptome sequencing (RNAseq) as "hints" for the location and structure of genes, further increasing the accuracy of *ab initio* gene prediction (Stanke *et al.*, 2008). Accuracy of gene prediction is assessed by comparing predicted genes to known gene sequence and identifying sensitivity and specificity of predictions: Sensitivity is the proportion of real genes that were predicted and specificity is the proportion of genes that were predicted correctly (Picardi and Pesole, 2010).

Finally, functional annotations are provided for predicted gene models. The annotation program Blast2GO is representative of a genome annotation pipeline (Conesa *et al.*, 2005, Gotz *et al.*, 2008). It identifies genome ontology (GO) terms (functional annotations) for predicted proteins by their BLAST similarity to known proteins and supports this with InterProScan searches to recognise known domains within each predicted protein (Altschul *et al.*, 1990, Quevillon *et al.*, 2005, Gotz *et al.*, 2008). The accuracy of functional annotation is assessed experimentally using techniques such as gene silencing or transient gene expression (Winnenburg *et al.*, 2008). Once gene functions are verified they are stored on databases such as those for particular organisms e.g. the *Saccharomyces* Genome Database (Cherry *et al.*, 2012); or biological interaction e.g. the Plant Host Interaction Database for genes involved in plant pathogenicity (Winnenburg *et al.*, 2008).

NGS technology is allowing research groups to perform whole genome sequencing to investigate differences within and between closely related fungi, such as assessing intraspecific variation within *Magnaporthe oryzae* (Xue *et al.*, 2012), determining of lineage specific regions within *Fusarium oxysporum* (Ma *et al.*, 2010) and identifying niche differentiation in closely related *Colletotrichum* species through differences in their interactions with host plants (O'Connell *et al.*, 2012). Generation of genomic resources for *A. alternata* will allow deeper investigation into this taxonomically complex group. This is an active area of research highlighted by the recent publication of the *A. arborescens* genome (Hu *et al.*, 2012) and with more *Alternaria* sp. genomes being prepared for publication (Dr. B. Pryor; personal communication).

OBJECTIVES AND OUTLINE OF THESIS

The primary objective of this study was to characterise the *Alternaria alternata* species group. Particular focus was put on understanding the pathogens *A. mali* and *A. gaisen*, responsible for leaf spot diseases of apple and pear and of phytosanitary importance in Europe. Understanding evolutionary relationships is important in defining the genetic and biological characteristics associated with plant pathogens in this species group. This can inform management strategies and facilitate the development of reliable detection tools for important plant diseases.

Firstly, **evolutionary relationships** within the *A. alternata* species group were established using a phylogenetic approach based on functional genes (Chapter 3). Following this, **morphological variation** within the *A. alternata* was studied (Chapter 4), allowing the association of morphological characters with phylogenetic clades identified in Chapter 3. The **presence of toxin-synthesis genes**, required for the production of host-selective toxins in apple and pear pathotypes, was established within *A. alternata* isolates (Chapter 5). The presence of different mating type genes in isolates was used to assess **evidence for recombination** within the *A. alternata* species group (Chapter 6). **Whole genome sequencing** was performed on 12 *A. alternata* isolates to further investigate presence of toxin genes (Chapter 5) and to determine whether *A. alternata* possess genes required for meiotic recombination (Chapter 6).

The general methods used in this study are presented in Chapter 2, including those for whole genome sequencing. Detailed descriptions of specialised techniques are presented in the relevant chapters (Chapters 3-6).

Finally, the main findings of the thesis are presented in Chapter 7, where areas of future research are also discussed and a framework for the identification of *A. alternata* pathogens is outlined.

CHAPTER 2

GENERAL METHODS

University of Warwick *Alternaria* culture collection

All work was performed using the University of Warwick (UoW) culture collection of 116 *Alternaria* isolates (Table 2.1). Isolates were obtained from either the Food and Environmental Research Agency (FERA), the private collection of Prof. E.G. Simmons (isolate names starting "EGS"), or from Dr. R.G. Roberts (starting "RGR") at the Tree Fruit Research and Extension Centre (TFREC), a United Sates Department of Agriculture (USDA) laboratory in Wenatchee, Washington. Two additional isolates, *HA-1* and *O-159*, were received from Dr. Philippe Gannibal at the All Russian Institute of Plant Protection. The host of an isolate was assumed to be the material it was isolated from. When known, the supplier of each isolate provided this information. Due to a lack of reliable information for the geographic origin of the FERA isolates this information has not been included.

Isolates provided by E.G. Simmons were reference isolates for *A. alternata*, *A. arborescens*, *A. gaisen*, *A. mali* and *A. tenuissima*. These isolates formed the basis for the morphological species descriptions as presented in Simmons (2007). Isolate *FERA 1410* originated from the Centraalbureau voor Schimelcultures (CBS603.78) and was considered to be a representative isolate of *A. alternata*.

Table 2.1*a* *Alternaria* isolates used in this study: Host is indicated where known. Use of isolate in phylogenetic study (Chapter 3), morphological analysis (Chapter 4) testing for toxin genes (Chapter 5) and mating type genes (Chapter 6) is also shown.

Isolate	Host		Reference for taxon	Phylogenetics	Morph. analysis	Toxin genes	Mating type
FERA 39	Schefflera	*Schefflera sp.*			√	√	
FERA 156					√	√	
FERA 326	Asian pear	*Pyrus pyrifolia*		√	√	√	√
FERA 348	Apple	*Malus domestica*		√	√	√	√
FERA 469	Mandarin orange	*Citrus reticulata*		√	√	√	√
FERA 478	Strawberry	*Fragaria sp.*		√	√	√	√
FERA 479	Strawberry	*Fragaria sp.*		√	√	√	√
FERA 482	Strawberry	*Fragaria sp.*		√	√	√	√
FERA 483					√	√	√
FERA 484				√	√	√	√
FERA 488	Peach	*Prunus persica*		√	√	√	√
FERA 538	Asian pear	*Pyrus pyrifolia*		√	√	√	√
FERA 540	Pear	*Pyrus sp.*		√	√	√	√
FERA 612	Brussel sprout	*Brassica oleracea*			√	√	√
FERA 631	European pear	*Pyrus communis*		√	√	√	√
FERA 632	Pear	*Pyrus sp.*		√	√	√	√
FERA 634	Apple	*Malus domestica*		√	√	√	√
FERA 635	Apple	*Malus domestica*		√	√	√	√
FERA 647	Pear	*Pyrus sp.*		√	√	√	√
FERA 648	Pear	*Pyrus sp.*		√	√	√	√
FERA 650	Pear	*Pyrus sp.*		√	√	√	√
FERA 675	Pear	*Pyrus pyrifolia*		√	√	√	√
FERA 678	Apple	*Malus domestica*		√	√	√	√
FERA 679	Apple	*Malus domestica*		√	√	√	√
FERA 680	Busy lizzie	*Impatiens walleriana*			√	√	
FERA 681	Pear	*Pyrus sp.*		√	√	√	√
FERA 704	Pear	*Pyrus pyrifolia*		√	√	√	√
FERA 743	Apple	*Malus domestica*		√	√	√	√
FERA 800					√	√	
FERA 802					√	√	
FERA 803					√	√	
FERA 805					√	√	
FERA 833	Apple	*Malus domestica*		√	√	√	√
FERA 840					√	√	
FERA 1082	Apple	*Malus domestica*		√	√	√	√
FERA 1105				√	√	√	√
FERA 1164	Apple	*Malus domestica*		√	√	√	√
FERA 1165	Apple	*Malus domestica*		√	√	√	√
FERA 1166	Apple	*Malus domestica*		√	√	√	√
FERA 1167	Apple	*Malus domestica*			√	√	
FERA 1168	Apple	*Malus domestica*			√	√	
FERA 1169	Apple	*Malus domestica*		√	√	√	√
FERA 1170	Apple	*Malus domestica*		√	√	√	√
FERA 1171	Apple	*Malus domestica*		√	√	√	√
FERA 1172	Apple	*Malus domestica*		√	√	√	√
FERA 1173	Apple	*Malus domestica*			√	√	
FERA 1174	Apple	*Malus domestica*			√	√	
FERA 1175	Apple	*Malus domestica*			√	√	
FERA 1176	Apple	*Malus domestica*			√	√	
FERA 1177	Apple	*Malus domestica*		√	√	√	√
FERA 1184	Apple	*Malus domestica*			√	√	
FERA 1307	Carrot	*Daucus carota*			√	√	
FERA 1308	Carrot	*Daucus carota*			√	√	
FERA 1310	Apple	*Malus domestica*		√	√	√	√
FERA 1410			*A. alternata*	√	√	√	√
FERA 1490	Strawberry	*Fragaria sp.*		√	√	√	√
FERA 1491	Strawberry	*Fragaria sp.*		√	√	√	√
FERA 1512	Chrysanthemum	*Dendrathema sp.*		√	√	√	√

Table 2.1*b* *Alternaria* isolates used in this study: Host is indicated where known. Use of isolate in phylogenetic study (Chapter 3), morphological analysis (Chapter 4) testing for toxin genes (Chapter 5) and mating type genes (Chapter 6) is also shown.

Isolate	Host		Reference for taxon	Phylogenetics	Morph. analysis	Toxin genes	Mating type
FERA 1513	Chrysanthemum	*Dendrathema sp.*			√	√	
FERA 1518	Tomato	*Solanum lycopersicum*		√	√	√	√
FERA 1519	Cotton	*Gossypium sp.*		√	√	√	√
FERA 1520	Lettuce	*Lactuca sp.*		√	√	√	√
FERA 1521	Apricot	*Prunus armeniaca*		√	√	√	√
FERA 1522	Kiwi	*Actinidia sp.*		√	√	√	√
FERA 1523	Watermelon	*Citrullus lanatus*		√	√	√	√
FERA 1539	Walnut	*Juglans regia*		√	√	√	√
FERA 1551	Apple	*Malus domestica*		√	√	√	√
FERA 2038	Tomato	*Solanum lycopersicum*		√	√	√	√
FERA 2040	Apple	*Malus domestica*		√	√	√	√
FERA 2041	Apple	*Malus domestica*		√	√	√	√
FERA 2042	Apple	*Malus domestica*			√	√	
FERA 2043	Apple	*Malus domestica*		√	√	√	√
FERA 2044	Apple	*Malus domestica*		√	√	√	√
FERA 2103	Citrus	*Citrus sp.*		√	√	√	√
FERA 2277	Busy lizzie	*Impatiens walleriana*		√	√	√	√
FERA 2280	Busy lizzie	*Impatiens walleriana*		√	√	√	√
FERA 2639	Brassica	*Brassica sp.*			√	√	
FERA 2646	Sweet potato	*Ipomoea batatas*			√	√	√
FERA 2712	Citrus	*Citrus sp.*		√	√	√	√
FERA 3250				√	√	√	√
FERA 9205	Salad rocket	*Eruca vesicana*			√	√	
FERA 9285	Busy lizzie	*Impatiens walleriana*		√	√	√	√
FERA 15182	Potato	*Solanum tuberosum*			√	√	
FERA 15968	Potato	*Solanum tuberosum*		√	√	√	√
FERA 20730						√	
FERA 21677	Pear	*Pyrus sp.*		√	√	√	√
FERA 23766	Asian pear	*Pyrus pyrifolia*		√	√	√	√
FERA 24350	Asian pear	*Pyrus pyrifolia*		√	√	√	√
EGS 34.015	Carnation	*Dianthus sp.*	*A. alternata*	√	√	√	√
EGS 34.016	Peanut	*Arachis sp.*	*A. tenuissima*	√	√	√	√
EGS 38.029	Apple	*Malus domestica*	*A. mali*	√	√	√	√
EGS 39.128	Tomato	*Solanum lycopersicum*	*A. arborescens*	√			√
EGS 90.0512	Asian pear	*Pyrus pyrifolia*	*A. gaisen*	√	√	√	√
RGR 97.0007	Apple	*Malus domestica*		√	√	√	√
RGR 97.0008	Apple	*Malus domestica*		√	√	√	√
RGR 97.0009	Apple	*Malus domestica*		√	√	√	√
RGR 97.0010	Apple	*Malus domestica*		√	√	√	√
RGR 97.0011	Apple	*Malus domestica*		√	√	√	√
RGR 97.0012	Apple	*Malus domestica*		√	√	√	√
RGR 97.0013	Apple	*Malus domestica*		√	√	√	√
RGR 97.0014	Apple	*Malus domestica*		√	√	√	√
RGR 97.0015	Apple	*Malus domestica*		√	√	√	√
RGR 97.0016	Apple	*Malus domestica*		√	√	√	√
RGR 97.0017	Apple	*Malus domestica*		√	√	√	√
RGR 97.0018	Apple	*Malus domestica*		√	√	√	√
RGR 97.0019	Apple	*Malus domestica*		√	√	√	√
RGR 97.0020	Apple	*Malus domestica*		√	√	√	√
RGR 97.0021	Apple	*Malus domestica*		√	√	√	√
RGR 97.0022	Apple	*Malus domestica*		√	√	√	√
RGR 97.0024	Apple	*Malus domestica*		√	√	√	√
RGR 97.0025	Apple	*Malus domestica*		√	√	√	√
RGR 97.0026	Apple	*Malus domestica*		√	√	√	√
RGR 97.0027	Apple	*Malus domestica*		√	√	√	√
RGR 97.0067	Apple	*Malus domestica*		√	√	√	√
HA-1	Apple	*Malus domestica*		√		√	√
O-159	Apple	*Malus domestica*		√		√	√

General experimental techniques

Routine culturing

Routine culturing of *Alternaria* isolates was performed on 1% potato-dextrose agar (PDA) agar plates. These were made using the following recipe: 15 g agar powder (Agar-agar technical, Merck) and 0.23 g potato dextrose broth (Fluka, Sigma Aldrich) were dissolved in 1 l of distilled water and autoclaved for 20 minutes at 121 °C. Following this the solution was left to cool to approximately 60 °C and then poured into individual agar plates under sterile conditions. Agar plates were inoculated from 14-28 day old growing cultures or from cultures in long-term storage. Cultures were grown for 14-28 days at 23°C in 12 hour day/night cycles in an incubator (MLR-350/T, Sanyo). Inoculations were made from growing cultures by using the end of a sterilised wooden cocktail stick to touch a young, representative sporulating region of the culture and then stab-inoculate the new agar plate at four equidistant points.

Long term storage

Long-term storage of cultures was performed by freezing colonised filter paper. Ten 0.5 cm discs of sterile filter paper were placed on un-colonised 1% PDA agar plates. This was done in aseptically in a safety cabinet using forceps. Slight pressure was applied to each filter paper as it was placed to encourage adhesion to the plate. Agar plates were inoculated (as described culturing) and grown 23 °C in 12 hour day/night cycles in an incubator (MLR-350/T, Sanyo) for 14-21days. Once mycelium/spores could be seen emerging from the upper side of the filter paper then filter papers were removed and placed into sterile 2 ml eppendorfs. Sterile paper towel was used to cover each eppendorf, secured by an elastic band. Eppendorfs were then placed in a freeze drier (Modulyo, Edwards) and desiccated for 48-72hours. Following this, the paper towel was removed from eppendorfs, their lids sealed and they were stored until required at -20 ^{o}C.

Culturing for DNA extraction

Alternaria isolates were cultured in 1ml of potato dextrose broth (PDB; Fluka, Sigma Aldrich) in eppendorf tubes (2 ml) covered with a small amount of cotton wool and silver foil. Inoculation of the PDB was as performed with a cocktail stick as described for routine culturing above. These cultures were grown for 14-21 days at 23°C in 12 hour day/night cycles in an incubator (MLR-350/T, Sanyo). Mycelium was then removed, rinsed with sterile distilled water, and freeze-dried. This freeze-dried material was then used for DNA extraction.

DNA extraction

Freeze dried *Alternaria* mycelium was powdered by placing a small amount of material in a skirted 1ml sample tube with a tungsten bead and shaken in a Fastprep-24 (MP Biomedicals) machine. DNA extractions from were performed on this powdered mycelium with a DNeasy Plant Mini Kit (Qiagen), using the protocol for DNA extraction from plant material as described in the manufacturer's handbook.

PCR amplifications

PCR was used to amplify *Alternaria* DNA in 20 µl reactions using a range of primers (Table 2.2). PCR reaction mixtures consisted of 10µl redtaq (REDTaq ReadyMix PCR Reaction Mix, Sigma-Aldrich), 2 µl DNA, 1 µl of each primer, and purified water (Sigma-Aldrich) to make the volume up to 20 µl. Specific annealing temperatures were used for each primer pair (Table 2.2). Following the PCR reaction, amplicons were visualised using gel electrophoresis using 1% agarose (Fischer Scientific) gels in tris-acetate-EDTA buffer (Fisher Scientific), stained with 5 µl of gel red (Biotium, UK).

Sanger sequencing

DNA amplicons produced by PCR were sequenced using Sanger sequencing. Before sequencing was performed, PCR purification was performed to extract DNA from the PCR reaction mixture using a QIAquick PCR Purification Kit (Qiagen). 5 µl of DNA and 5 µl of 5 pM primer were submitted to the LIGHTRun sequencing service (provided by GATC) for sequencing in both directions, using the same primers as used for PCR (Table 2.2). Following sequencing, data was aligned, edited and saved as consensus sequences using SeqMan (Burland, 2000). Consensus sequences and sequences downloaded from Genbank were stored in .fasta file format. DNA sequences were imported into Geneious (Kearse *et al.*, 2012) where sequence alignment was performed using the ClustalW algorithm and refined using the Muscle algorithm.

Table 2.2 Primers and target gene loci used in PCR amplifications: Primers, target gene loci and annealing temperatures used in PCR amplifications in this study. Citations are shown as appropriate.

Genetic locus		Primer name	Primer sequence (5'-3')	Length (bp)	Annealing temp. (°C)	Amplicon length (approx. no. bp)	Source
ITS	Nuclear rDNA internal transcribed spacer	ITS1	TCCGTAGGTGAACCTGCGG	19	55	456	White *et al.*, (1990)
		ITS4	TCCTCCGCTTATTGATATGC	20			
EndoPG	Endopolygalacturonase gene	PG3	TACCATGGTTCTTTCCGA	18	50	485	Peever *et al.*, 2009
		PG2b	GAGAATTCRCARTCRTCYTGRTT	22			
Alta1	Major alternaria allergen gene	Alt-for	ATGCAGTTCACCACCATCGC	20	57	507	Lawrence *et al.*, (2011)
		Alt-rev	ACGAGGGTGAYGTAGGCGTC	20			
L152	Aegerolysin-like gene	L152-F1	GCACCAGAGTCCTGATTCCA	20	58	454	Roberts *et al.*, (2012)
		L152-R1	GTCAAAAATGCTGAAGCCATG	21			
MAT1-1-1	Mating type idiomorph MAT1-1 gene	AAM1-2	TCCCAAACTCGCAGTGGCAAG	21	57	576	Arie *et al.*, (2000)
		AAM1-3	GATTACTCTTCTCCGCAGTG	20			
MAT1-2-1	Mating type idiomorph MAT1-2 gene	AAM2-F	AAGGCTCCTCGACCGATGAA	20	57	271	Arie *et al.*, (2000)
		AAM2-R	CTGGGAGTATACTTGTAGTC	20			
AMT1	*AMT1* gene involved in the synthesis of *Alternaria* apple toxin	AMT-LinF1	TATCGCCTGGCCACCTACGC	20	65	458	Johnson *et al.*, (2000)
		AMT-LinR	TGGCCACGACAACCCACACATA	20			
AMT2	*AMT2* gene involved in the synthesis of *Alternaria* apple toxin	AMT-2f2	GTTGCAGAATCGCAAACTCA	20	57	613	Roberts *et al.*, (2012)
		AMT2r2	GGCTCTTGGTCTCAAATCCA	20			
AKT1	*AKT1 gene involved in the synthesis of Alternaria pear toxin*	AKT-1F	GCTCGACTGGCCTCAAAAGC	20	65	310	Roberts *et al.*, (2012)
		AKT-1R	AGAACCAGGCGCATCGGTTA	20			
AKT2	*AKT2 gene involved in the synthesis of Alternaria pear toxin*	AKT-2F	GCCGGGAGAGACGAGGAAAG	20	65	514	Roberts *et al.*, (2012)
		AKT-2R	CATGCGACCTCAATCGATCG	20			
MS432	Gene present on FUNYBASE: Unknown function	MS432-A-F	CAAGCTCTCTCTTTCGCGTC	21	62	355	N/A
		MS432-A-R	GAGGGAGGCCATGTTCTGCTG	21			
MS550	Gene present on FUNYBASE: Phosphatidylglycerolphosphate synthase	MS550-A-F	CAGACGCCTGCCGAGTTTTAT	21	60.5	537	N/A
		MS550-A-R	CCTTCGTTGATGCGTTTAGG	20			
MS578	Gene present on FUNYBASE: Subunit of DNA polymerase zeta	MS578-A-F	CTGTAGAGAGTATTGAAGATC	21	54.8	407	N/A
		MS578-A-R	AGGTGTCTTGGGGCCTTCTTG	21			

Genome sequencing of 12 *Alternaria* isolates

Genome sequencing was performed on twelve *Alternaria* isolates during the course of this study. Isolates were chosen to represent strains from apple and pear, from different phylogenetic clades (as determined from the multi-locus phylogeny in Chapter 3) and possessing different toxin-synthesis genes (as indicated by PCR screens in Chapter 5). Transcriptome sequencing (RNAseq) was performed on two of these isolates to aid gene prediction (Fig. 2.3). Assembled genomes and predicted genes were used for *in silico* analysis of toxin genes (Chapter 5) and identification of conserved meiotic genes (Chapter 6) in *Alternaria* spp..

Culturing for genome sequencing

Single spore isolations were made for the twelve *Alternaria* isolates from growing cultures. Isolations were then put into long-term storage until needed (as described earlier). A disk of filter paper from long-term storage was used to inoculate conical flasks containing 250 ml PDB (see routine DNA extraction), which were then sealed with sterile cotton wool in the neck of the flask and covered by silver foil. These liquid cultures were incubated for 14 days at room temperature before mycelium was separated from the PDB by filtering through a Buchner funnel and freeze dried (as described for routine DNA extraction) for 72 hrs. Desiccated mycelium was stored at -80 ^{o}C until required.

Sequencing Protocol for isolate FERA 1166 using an Illumina GA2 genome analyser

DNA extraction was performed on freeze-dried mycelium using a cetyltrimethylammonium bromide (CTAB) extraction protocol. This was taken from Li *et al.* (1994), modified by D.J. Barbara (University of Warwick) and presented below (also presented in PhD theses by Vagany (2012) and Baroncelli (2012)): 250 mg of freeze-dried mycelium was homogenised with 1 g of sterile sand using a chilled, sterile pestle and mortar. The ground mycelium / sand was transferred into a 50 ml Falcon tube and mixed by inversion with 15 ml extraction buffer (8.18 g NaCl

dissolved in 70 ml dH_2O with 5 ml 2M pH 8.0 Tris-Cl, 4 ml 0.5M pH 8.0 EDTA, 7.4 ml 10% CTAB, 13.6 ml dH2O, 2 g polyvinylpyrrolidone 40 (PVP-40) and 0.5 ml 2-mercaptoethanol). The sample was incubated at 60°C for 30 minutes and mixed gently by inverting occasionally. 15 ml of the chloroform/penta-1-ol wash (96 ml chloroform and 4 ml penta-1-ol) was added to the sample, which was mixed by gentle inversion for 10 minutes followed by centrifugation. Centrifugation steps were carried out at 1500 G (2914 rpm) for 10 minutes at 20°C, unless stated otherwise. The aqueous phase was transferred into a sterile 50 ml Falcon tube and 15 ml of chloroform/penta-1-ol wash was added again followed by gentle mixing by inversion for 10 minutes then centrifuged. The aqueous phase was transferred into a sterile 50 ml Falcon tube and centrifuged again. 0.6 volumes of cold (-20°C) isopropanol was added to aqueous phase and mixed by inverting tube gently. The sample was incubated at room temperature for 2 hours before centrifuging at 460 G (1500 rpm) for 2 minutes at 20°C. The supernatant was then removed and the pellet dissolved in 10 ml wash buffer (66 ml 100% ethanol and 34 ml 0.1M NaCl) by gentle inversion for 20 minutes followed by centrifugation. Supernatant was removed and rinsed again in 10 ml wash buffer then was centrifuged. The supernatant was removed and the pellet was air dried for 20 minutes. The pellet was then dissolved in 1.5 ml Tris-EDTA (10mM pH 8.0 Tris-Cl and 1 mM EDTA) and transferred into a 1.5 ml Eppendorf tube using a pipette tip with the end cut off. The sample was centrifuged at maximum speed for 5 minutes and the supernatant was transferred into a 1.5 ml Eppendorf tube using a pipette tip with the end cut off.

RNase treatment was applied to degrade residual RNA. A volume containing approximately 10 µg of DNA was taken and 1 µl of RNase (RNase A 7 U/µl, Qiagen) added for every 25 µl of the DNA sample. The sample was incubated at room temperature for 30 minutes before being desalted using a Qiaex II gel extraction/desalting kit (Qiagen); manufacturer's guidelines were followed, except that the final elution was performed using 30 µl of buffer EB from a QIAQuickPCR purification kit (Qiagen).

Extracted gDNA was tested for contamination through amplification and sequencing using the ITS and endopolygalacturonase (*endoPG*) regions (as described for routine PCR). Sequence data was compared to that generated in Chapter 3.

The concentration of the extracted gDNA was measured using a Qubit Flurometer (Invitrogen) and adjusted to a concentration of 20 $ng.\mu l^{-1}$ before being submitted to the genomics service at the School of Life Sciences, University of Warwick for genomic library preparation and sequencing. The genomics service produced 200 bp cDNA libraries using a TrueSeq protocol (TrueSeq Kit, Illumina) and sequenced these libraries using 76 bp paired-end reads on an Illumina GA2 Genome Analyser. The DNA was sequenced in a multiplex reaction with another unrelated genome (*Fusarium oxysporum*: *Fus2*, sequenced in Vagany (2012)) using half the capacity of a single lane.

Sequencing Protocol for 11 isolates using an Illumina MiSeq

DNA extraction for the other eleven isolates was performed using a GenElute Plant DNA Miniprep Kit (Sigma). The manufacturers protocol was used with the following modifications: The volume of lysis solutions (PartA and PartB) were doubled; an RNase digestion step was performed as suggested in the manufacturers protocol; twice the volume of precipitation solution was added; elution was performed using elution buffer EB (Qiagen).

Extracted gDNA was tested for contamination through amplification and sequencing using of the ITS and *endoPG* regions (as described for routine PCR). Sequence data was compared to that generated in Chapter 3.

Genomic libraries were prepared using a Nextera Sample Preparation Kit (Illumina), following the manufacturers protocol. A fragment analyser was used to identify and select for libraries with high representation of DNA fragments 600-1000 bp in length. Once libraries had been generated for each isolate, samples were sequenced in multiplex (five or six libraries per lane) on a MiSeq Benchtop Analyser (Illumina) using 250 bp, paired end reads. Libraries were re-sequenced until a minimum of 20 times "estimated coverage" was achieved for each isolate (Table 2.3). "Estimated coverage" was determined by number of bp of sequence generated, minus the number of bp errors (as predicted using Geneious (Kearse *et al.*, 2012)) and dividing this

number by the estimated size of the *Alternaria* genome (35 Mb).This work was performed at East Malling Research under supervision by Dr. R. Harrison.

De novo Genome Assembly

De novo genome assemblies were generated from sequencing data for each isolate using the program Velvet (Zerbino and Birney, 2008) on the computer cluster at East Malling Research (Table 2.3). The parameters used by Velvet for assembly of the *FERA 1166* genome (from 75 bp paired-end reads) used a *hash-length* of 41 bp, an *insert length* of 250 bp, a *coverage cut-off* of five, and a *minimum final contig length* of 200bp. Due to the MiSeq read lengths being longer (250 bp), parameters were modified for the other 11 genomes, by using a *hash length* of 41 bp, an *insert length* of 700 bp and a *minimum final contig length* of 1500 bp. The parameter for *estimated genome coverage* was set to that of the "estimated coverage" calculated above.

The quality of genome assemblies were observed (Table 2.3). In general, genome assemblies showed N50 values greater than 1Kb, with similar coverage to that estimated and had less than 50 contigs in N50 (Table 2.3), indicating good assemblies that could be used as a basis for gene prediction, and use in Chapters 5 and 6.

Table 2.3 Isolates used and data generated from genome sequencing: Details of twelve strains used for genome sequencing including material isolations were originally made from and their phylogenetic haplotype (as determined in Chapter 3: Fig. 3.2). Sequence data generated and details of de novo assemblies are shown, including commonly used measures for quality of genome assembly.

Strain			Sequence Data Generated			Assembly details				Measures of assembly quality		
Name	Isolated from	Phylogenetic clade	No. nucleotides	Expected errors	Estimated coverage	Assembled genome (Mb)	No. contigs	Largest contig (Mb)	Coverage of N50 contigs	N50 (Mb)	Mean coverage	No. contigs in N50
FERA 1166	*Malus domestica*	*2b*	$2.01x10^9$	$14.8x10^6$	57.0	33.3	366	3.79	18	0.74	77	13
FERA 635	*Malus domestica*	*2a*	$1.19x10^9$	$10.9x10^6$	33.8	35.2	964	0.45	19	0.09	24.7	113
FERA 648	*Pyrus sp.*	*2b*	$2.01x10^9$	$17.5x10^6$	56.9	33.4	241	1.35	40	0.49	52.1	23
FERA 650	*Pyrus sp.*	*3*	$1.47x10^9$	$28.6x10^6$	41.2	33.6	452	0.67	25	0.17	36.3	59
FERA 675	*Pyrus pyrifolia*	*1a*	$1.38x10^9$	$11.3x10^6$	39.0	33.5	294	1.53	28	0.40	27.4	26
FERA 743	*Malus domestica*	*2a*	$1.80x10^9$	$21.6x10^6$	50.8	35.2	492	1.73	31	0.43	48.7	22
FERA 1082	*Malus domestica*	*2d*	$0.91x10^9$	$16.0x10^6$	25.6	33.7	607	0.46	16	0.12	24.4	89
FERA 1164	*Malus domestica*	*2e*	$1.16x10^9$	$14.6x10^6$	32.6	34.5	413	1.45	23	0.25	35.6	40
FERA 1177	*Malus domestica*	*2e*	$2.63x10^9$	$16.3x10^6$	74.8	34.8	612	1.36	47	0.28	94.2	40
FERA 24350	*Pyrus pyrifolia*	*2a*	$1.37x10^9$	$23.2x10^6$	38.4	32.9	219	1.53	28	0.39	31.1	25
RGR 97.0013	*Malus domestica*	*1a*	$1.41x10^9$	$15.1x10^6$	39.7	33.5	313	1.26	29	0.45	35	23
RGR 97.0016	*Malus domestica*	*1a*	$1.06x10^9$	$11.4x10^6$	29.9	33.4	330	0.31	22	0.27	28	36

Gene prediction using evidence from transcriptome sequencing

Transcriptome sequencing (RNAseq) was performed on an apple pathotype isolate *FERA 1166* and a pear pathotype isolate *FERA 650*. The evidence generated from this informed gene prediction algorithms, which were used to predict genes on isolates *FERA 1166*, *FERA 650* and on a third isolate from which evidence was not generated *FERA 675*.

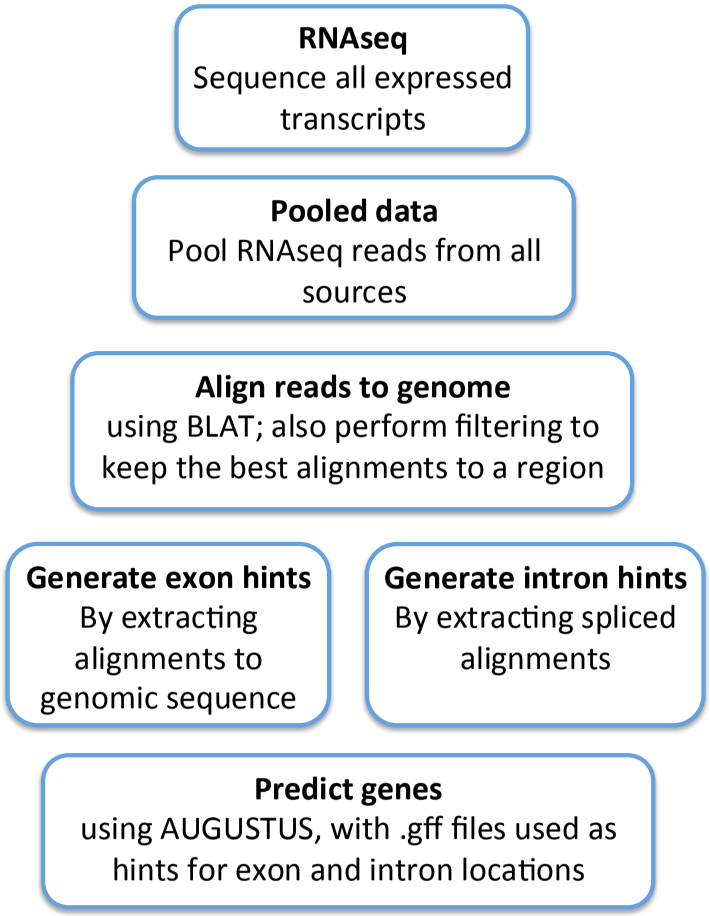
FERA 1166 and *FERA 650* were each grown in four different culturing media, which were PDB, 1%PDB, potato carrot broth (PCB) and V8 juice broth (V8B). The recipe for making PDB was the same as described for routine DNA extraction, and 1%PDB also used this recipe, except 1% of the recommended weight of PDB powder was used. The protocol for making PCB and V8B were the same as described in Simmons (2007) for making potato carrot agar (PCAgar) and V8 juice agar, with the exception that agar was not added to the recipe: To make V8B, 200 ml of V8 juice (Campbell's) was diluted in 800 ml of distilled water and autoclaved; to make PCB, 20 g of white potato and 20 g of carrot were finely sliced and autoclaved in 200 ml of water, which was then strained through a fine muslin cloth and this resultant solution made up to 1 l with sterile water before being re-autoclaved.

Cultures were grown in conical flasks containing 250 mls of liquid medium for 14 days at room temperature (as when culturing for genome sequencing). Mycelium for the four cultures of *FERA 1166* (PDB, 1%PDB, PCB and V8B) and four cultures of *FERA 650* was separated from liquid medium by filtering through a Buchner funnel before freeze drying for 72 hrs and stored at -80 ^{o}C. mRNA extraction was performed on freeze-dried mycelium using RNeasy Plant RNA extraction Kit (Qiagen), following the recommended protocol. mRNA samples were stored at -80^{o}C, or on dry ice at all times to minimise mRNA degradation.

Concentration and quality of the four mRNA samples for both strains were assessed using a Bioanalyzer (Agilent Technologies). mRNA from the 1%PDB culture of *FERA 650* showed evidence of RNase contamination and was not used further. The remaining three samples (PDB, PCB and V8B) for isolate *FERA 650* were adjusted to a concentration of 20 ng.μl^{-1} and pooled together in equal volumes. The four mRNA samples for isolate *FERA 1166* were also adjusted and pooled. Both pooled samples

were submitted to a genomics service (School of Life Sciences, University of Warwick) where 200 bp cDNA libraries were prepared using a TrueSeq protocol (TrueSeq Kit, Illumina). These libraries were sequenced in multiplex on a MiSeq (Illumina) at East Malling Research, using 200 bp paired end reads.

RNAseq reads from *Alternaria* isolates *FERA 1166* and *FERA 650* were pooled and used to perform evidence-based *de novo* gene prediction on three genomes: *FERA 1166*, *FERA 650* and *FERA 675* (Fig. 2.1). *Ab initio* prediction was performed using the computer cluster at East Malling Research to run a pipeline of bioinformatic tools suggested in the documentation (readme.rnaseq.html) provided with AUGUSTUS version 2.5.5 (Stanke *et al.*, 2008). This involved, first using the program BLAT (Kent, 2002) to align all RNA-seq reads to the target genome. Following this this the Perl script filterPSL.pl (provided with Augustus 2.5.5) was used to filter-out all aligned reads that had high identity and to remove sequences that poorly match the target genome. Filtered alignments were to determine potential coding sequence through identifying the best alignments to genomic sequence using the program aln2wig (provided with Augustus 2.5.5) from which hints for exons (coding regions) were generated using the Perl script wig2hints.pl (provided with Augustus 2.5.5). The filtered alignment was also used to identify locations of spliced alignments using the Perl script blat2hints.pl (provided with Augustus 2.5.5), which were used as hints for introns. Both intron and exon hints were used to provide evidence for the location and structure of genes in AUGUSTUS (Stanke *et al.*, 2008), which was run using parameters trained to fungal pathogen *Fusarium graminearum* (Fig. 2.3).



		FERA 1166	*FERA 650*	*FERA 675*
No. reads		12.8×10^6	13.2×10^6	
No. pooled reads used for hints			28.0×10^6	
No. of reads in Augustus hints	**Hints for genes**	1.9×10^6	1.8×10^6	2.0×10^6
	Hints for introns	36705	34940	38835
No. predicted genes		**14105**	**13498**	**13223**

Figure 2.1 Pipeline of de novo gene prediction: As used to predict genes in strains *FERA 1166*, *FERA 650* and *FERA 675*. The number of reads generated from RNAseq and their use in gene prediction for the three isolates are shown.

CHAPTER 3

PHYLOGENETIC ANALYSIS OF *ALTERNARIA ALTERNATA*

3.1. INTRODUCTION

The *Alternaria alternata* species group is largely phylogenetically unresolved. Successful application of molecular techniques is dependent upon identifying loci with appropriate variability to show resolution within the species group. Principles of phylogenetic species recognition and genealogical concordance can be applied to identify species boundaries. Furthermore phylogenetics can also be used to assess whether the isolates used in this study are genetically representative of the *A. alternata* species group.

Protein coding genes as phylogenetic loci

Ribosomal DNA loci are considered the most appropriate loci for fungal barcoding, particularly the internal transcribed spacer (ITS) region, which has been proposed as the universal fungal barcoding marker (Schoch *et al.*, 2012). Other loci such as the 18S nuclear ribosomal small subunit (SSU) and the 28S nuclear large ribosomal subunit have also shown promise as barcoding loci (Schoch *et al.*, 2012). However, protein-coding genes have indicated greater potential than rDNA genes to resolve evolutionary relationships within the Ascomycetes (Schoch *et al.*, 2009a). Protein coding genes are increasingly being used to resolve evolutionary relationships in closely related species. Commonly used loci include the translation elongation factor 1-α (*TEF*) locus, RNA Polymerase II (*RPB2*), glyceraldehyde-3-phosphate dehydrogenase (*gpd*) and the Endopolygalacturonase (*endoPG*) locus.

Primers with broad specificity within the fungi were developed as part of the "Assembling the Fungal Tree of Life" (AFTol) project, including primers for the RPB2 and TEF loci (James *et al.*, 2006). The *RPB2* locus has been shown to possess population and species level variability in other genera. The RPB2 locus has been used in combination with the *TEF* locus to resolve taxa at the species level, including establishment of a species identification database in *Fusarium* (O'Donnell *et al.*, 2010). These two loci have been used to resolve the Dothideomycete Class, along with the 18S nuclear ribosomal subunit (SSU*)* and 28S large ribosomal subunit (LSU)

loci (Schoch *et al.*, 2006). The *gpd* locus has been used to investigate phylogenetic relationships in the Dothideomycetes, in *Cochliobolus* and *Stemphylum* (Berbee *et al.*, 1999, Camara *et al.*, 2002).

Endopolygalacturonases are involved in degradation and remodelling of plant cell walls. These enzymes are used by phytopathogenic fungi to aid penetration and colonisation of plant cell tissues (De Lorenzo *et al.*, 2001), and as such are involved in interactions with plant-produced endopolygalacturonase inhibiting proteins. These specific interactions put evolutionary pressure on endopolygalacturonase to differentiate (Federici *et al.*, 2001). A locus based upon the endopolygalacturonase gene region, known as *endoPG* has shown variability within the small-spored *Alternaria*. This variability has also been observed in polygalacturonase genes for other fungal genera, such as *Fusarium oxysporum* (Hirano and Arie, 2009). The *endoPG* locus has more accessions on Genbank for individuals in the *A. alternata* species group than any other locus (as of December 2013).

Loci designed for *Alternaria* phylogenetics

Novel loci may be developed specifically for phylogenetics in a system. Primers may be limited to the system being studied. A number of phylogenetic loci (*Alta1*, *OPA1-3*, *OPA10-2* and *L152*) have been specifically developed for phylogenetics within the *Alternaria* genus and for closely related taxa.

The *Alternaria* major allergen 1 (*Alta1*) is one of a diverse set of proteins present in *A. alternata* responsible for inducing allergenic responses in humans (Achatz *et al.*, 1995). A homolog of the *Alternaria* major allergen 1 gene, present in *Alternaria brassicicola*, has previously been shown to be highly up-regulated during the infection process on *Arabidopsis thaliana*, suggesting the gene may be involved in plant pathogenesis (Cramer and Lawrence, 2004). Primers were designed for a 458bp region of the gene, referred to as the *Alta1* locus, which showed resolution between species within the *Alternaria* genus (Hong *et al.*, 2005). The locus was estimated to contain 3.5 times more parsimoniously informative sites than the *gpd* locus (Hong *et al.*, 2005). The *Alta1* locus was used in recent studies to identify evolutionary

relationships in *Alternaria* genus, showing resolution between *Alternaria* species outside of the *A. alternata* species group (Lawrence *et al.*, 2011). This locus has the second greatest number of accessions on Genbank within the *A. alternata* species group.

Uncharacterised loci *OPA1-3* and *OPA10-2* have been used to resolve taxa within the small-spored *Alternaria* taxa (Andrew *et al.*, 2009). Phylogenies constructed from these loci had incongruent tree topologies between *OPA10-2* and *OPA1-3* (Andrew *et al.*, 2009). Incongruence observed between these loci was not examined or used as a basis for species recognition. Use of these loci has mainly been in the citrus pathosystem where they have shown similar resolution to *endoPG* (Peever *et al.*, 2004, Peever *et al.*, 2005).

Exploration of differentially expressed genes between taxa has been used to identify novel loci for phylogenetics. A study investigating differential expression of *A. gaisen* genes under light and dark conditions led to the identification of *L152* a highly variable genetic locus on a gene containing an aegerolysin domain (Roberts *et al.*, 2011). Aegerolysins have a broad range of biological activities in fungi, bacteria and plants (Berne *et al.*, 2009). In fungi they are best understood as virulence factors in *Aspergillus fumigatus* (Rementeria *et al.*, 2005). The *L152* locus showed high resolution between six small spored *Alternaria* taxa (Roberts *et al.*, 2011). This conclusion was drawn from only 11 isolates and therefore further work needs to be performed on this locus to verify its wider use in the *Alternaria* and other taxa.

Recent studies into the *A. alternata* species group have attempted to resolve taxa to formalise current taxonomic status (Lawrence *et al.*, 2013, Woudenberg *et al.*, 2013). In doing so, novel loci for phylogenetics were developed. Three such genes that showed phylogenetic utility within the *Alternaria* genus were actin, plasma membrane ATPase (ATPase), and calmodulin (Lawrence *et al.*, 2013).

Identifying new phylogenetic loci using genomic databases

Genome sequencing is allowing new possibilities for identifying novel loci for phylogenetics and diagnostics. Loci can be identified on genetic regions that show

diversity at a specific taxonomic level. Within the fungal kingdom 246 genes have been identified as present in a single copy in all fungi. Single copy genes are preferred as phylogenetic loci as multi-copy genes may be present in non-identical paralogs (Kiss, 2012). These 246 fungal genes are recorded on the publicly available Fungal Phylogenomic Database (FUNYBASE) as DNA and amino acid sequences as they appear in the *S. cerevisiae* genome.

A number of Dothideomycete genomes have been sequenced including *Pyrenophora tritici-repentis*, *Phaeospharea nodorum*, *Chochliobolus heterostrophus* and *A. brassicicola*. Genome sequence information can be used to identify highly variable loci between Dothideomycete species. The utility of commonly used barcoding loci can be assessed by comparing the variability between these closely related genomes. This provides an opportunity to identify novel phylogenetic loci that have greater levels of variability than loci previously used to perform phylogenetics on the *A. alternata* species group.

Collaboration with the Tree Fruit Research and Extension Centre

An extensive *Alternaria* culture collection is held at the Tree Fruit Research and Extension Centre (TFREC) in Wenatchee (Washington, USA). This culture collection has been compiled and maintained by R.G. Roberts, who through research with the United States Department of Agriculture (USDA), has worked extensively on *Alternaria* diseases. This culture collection contains many ex-type reference isolates, as described by E.G. Simmons (Simmons, 2007). Isolates from this culture collection have been studied in a number of publications by E.G. Simmons and R.G. Roberts (Simmons and Roberts, 1993, Roberts *et al.*, 2000, Roberts, 2001, Roberts, 2005, Roberts *et al.*, 2011). The culture collection contains many isolates from apple and pear as Wenatchee is a major apple growing region in the USA. Due to R.G. Roberts's expertise in morphological identification of *Alternaria* species, the TFREC culture collection has received cultures as part of requests for taxonomic identification. This has resulted in the culture collection representing accessions from a diversity of hosts and geographic locations. Unpublished data from this culture collection includes *L152* sequence data for 137 isolates. This data was generated by

R.G. Roberts and S. Reymond, following the development of the *L152* locus in Roberts *et al.* (2011). Permission has been given to use these sequences data in this study. Comparing the genetic diversity within the University of Warwick (UoW) *A. alternata* culture collection to TFREC resources should help establish whether the UoW culture collection represents the diversity found in the *A. alternata* species group.

3.2 AIMS

Although previous phylogenetic work has attempted to resolve the *A. alternata* species group, results have been inconclusive. Phylogenetics has failed to resolve sequence data for *A. gaisen*, *A. alternata*, *A. tenuissima* and *A. arborescens* morphological species. This chapter aims to use highly variable loci to identify evolutionary relationships within *A. alternata* isolates from the University of Warwick (UoW) culture collection.

The specific aims were as follows:

1. Identify loci from the literature showing high variability within the small-spored *Alternaria*.
2. Use the Fungal Phylogenomic Database (FUNYBASE) to identify new loci for phylogenetics within the *A. alternata* species group.
3. Identify evolutionary relationships within the *A. alternata* species group using single and multi-locus phylogenies constructed from the highly variable genetic loci identified above.
4. Assess whether the genetic diversity of the UoW culture collection is representative of the *A. alternata* species group by comparing *L152* genotypes in the UoW culture collection to those of the diverse set of isolates from the Tree Fruit Research and Extension Centre (TFREC).

3.3 MATERIALS AND METHODS

Identifying highly variable loci

The comparative use of 12 phylogenetic loci for resolving the *A. alternata* species group was assessed. This was done by comparing the variability (number of SNPs) within alignments of representative isolates within: 1) Four representative isolates of morphological species in the *A. alternata* species group (*A. alternata*, *A. arborescens*, A. *gaisen* and *A. tenuissima*); 2) between the *A. alternata* species group and a closely related species (*A. brassicicola*). The loci assessed included rRNA genetic regions (SSU, LSU and ITS) and single copy coding regions; some of which have been widely used for phylogenetics (*RPB2*, *TEF*, *gpd*) and some regions that have not been used widely outside of *Alternaria* (*Alta1*, *endoPG*, *L152*, Actin, ATPase and calmodulin). All of these loci have previously been used for phylogenetics within the *Alternaria* genus (Peever *et al.*, 2004, Roberts *et al.*, 2011, Lawrence *et al.*, 2013, Woudenberg *et al.*, 2013).

Representative isolates were used for morphological species *A. alternata*, *A. arborescens*, *A. tenuissima*, *A. mali*, *A. gaisen* and *A. brassicicola*. The strains used were those designated as the ex-type strain by Simmons (2007). Sequence data for each of these isolates was publically available from previous published studies and was downloaded from Genbank (Table.3.1). A second, non-ex-type, strain of *A. brassicicola* was used for five loci as sequence data was not available for the ex-type isolate (Table.3.1).

No Genbank accessions were available for the *A. brassicicola endoPG* region. A sequence was generated for this locus by performing a BLAST search upon the publicly available *A. brassicicola* genome (isolate *EGS 42.002*) and downloading the region showing highest identity to the other accessions downloaded for the *endoPG* locus.

Alignments of downloaded sequence data were made in Geneious (Kearse *et al.*, 2012) using the 'Muscle alignment' algorithm, followed by manual editing of the alignment. This was performed for all twelve loci. Alignments were exported to

MEGA 5.1 (Tamura *et al.*, 2011) where the numbers of single nucleotide polymorphisms (SNPs) were observed using the 'Highlight Variable Sites' function. The numbers of SNPs within the *A. alternata* species group were determined using the same alignment, with the *A. brassicicola* sequence unselected in the MEGA 5.1 alignment viewer.

Table 3.1 Genbank accessions used to determine the variability of 12 phylogenetic loci: Sequence data generated in previous studies and downloaded for this work is shown for six strains representing five *Alternaria* species.

Taxon	*A. alternata*	*A. arborescens*	*A. brassicicola*		*A. gaisen*	*A. tenuissima*	Sequenced in
Isolate	***EGS 34.016***	***EGS 39.128***	***EGS 42.002***	***EEB 2232***	***EGS 90.0512***	***EGS 34.015***	
SSU	KC584507	KC584509	KC584515		KC584531	KC584567	Woudenberg *et al.*, (2013)
LSU	DQ678082	KC584253	KC584259		KC584275	KC584311	
RPB2	KC584375	KC584377	KC584383		KC584399	KC584435	
ITS	AF347031	AF347033	JX499031		KC584197	AF347032	
TEF	KC584634	KC584636	KC584642		KC584658	KC584693	
gpd	AY278808	AY278810		AY278813	JQ646317	AY278809	Lawrence *et al.*, (2013)
***Alta*1**	AY563301	AY563303		AY563311	JQ646400	AY563202	
Actin	JQ671702	JQ671705		JQ671669	JQ671699	JQ671703	
ATPase	JQ671874	JQ671880		JQ671843	JQ671871	JQ671875	
Calmodulin	JQ646208	JQ646214		JQ646177	JQ646205	JQ646209	
EndoPG	AY295024	AY295028			AY295033	AY629223	Peever *et al.*, (2004)
L152	HQ238264	HQ238263	HQ238257		HQ238259	HQ238266	Roberts *et al.*, (2011)

Identify novel loci for phylogenetics using the genetic database FUNYBASE

Genes were identified in the Fungal Phylogenomic Database (FUNYBASE) that showed high variability between species in the Dothideomycete Class. These genes were used to identify loci for phylogenetics within the *A. alternata* species group.

Orthologs of 26 FUNYBASE genes were downloaded for six Dothideomycete fungi with publicly available genome sequence (January 2010): *A. brassicicola*, *Phaeospharea nodorum*, *Pyrenophora tritici-repentis*, *Cochliobolus heterostrophus*, *Mycosphaerella graminicola* and *Mycosphaerella fijiensis*. Sequence data was

downloaded as follows: Amino acid sequence, as found in the genome of *S. cerevisiae*, was available on the FUNYBASE database for each of the 26 genes. This was used as a query sequence to perform a BLAST search against each of the six Dothideomycete genomes using the tBLASTn algorithm. Genomic sequence for the orthologous gene, as identified by the tBLASTn, was downloaded for each Dothideomycete species. The Dothideomycete genomes were publicly available on the Joint Genome Institute (JGI) (Grigoriev *et al.*, 2012), or Broad institute genome databases (Broad, 2013), and the BLAST searches were performed using the web-server for websites. Genomic sequences for each gene were imported into sequence analysis software MEGA 5.1 (Tamura *et al.*, 2011) where sequence alignment was performed using the ClustalW algorithm. This was repeated for the 26 genes. Sequence data was trimmed at the point of the first and last conserved base in the alignment. The variability of each gene was determined by noting the length of the alignment and the number of SNPs in the alignment between all Dothideomycete taxa. The number of SNPs between A. *brassicicola* and its close relatives *P. tritici-repentis*, *P. nodorum* and *C. heterostrophus* was also noted.

Ten of these genes were selected for further analysis. For these genes, potential primer sites were identified within alignments of *A. brassicicola*, *P. tritici-repentis*, *P. nodorum* and *C. heterostrophus.* The positions of variable and conserved regions were noted. Primers were designed by eye in conserved regions either side of variable regions for 17 loci (Table 3.2). Three of these 17 loci were selected for phylogenetic analysis. These were chosen on the basis of them indicating high variability, suitability of primer sites and being of a length of approximately 500-700bp. The number of SNPs within each of the three loci was compared to highly variable loci from previous studies (described above). This was done by performing PCR on gDNA from ex-type isolates for *A. alternata* (*EGS 34.015*), *A. mali* (*EGS 38.029*), *A. gaisen* (*EGS 90.0512*) and *A. tenuissima* (*EGS 34.016*) using primers designed above (PCR conditions were the same as described in Chapter 2). Sanger sequencing was performed on the amplicons (as described in Chapter 2). The sequence data generated for these four ex-type isolates along with the sequence data already downloaded for *EGS 42.002* was aligned in MEGA 5.1 (Tamura *et al.*, 2011). The numbers of single nucleotide polymorphisms (SNPs) were observed using the *highlight variable sites* function. The numbers of SNPs within *A. alternata* species group isolates (*A.*

alternata, *A. tenuissima*, *A. mali* and *A. gaisen*) were determined using the same alignment, with the *A. brassicicola* sequence unselected in the MEGA 5.1 alignment viewer.

Table 3.2 17 primer pairs designed for highly variable loci within 10 FUNYBASE genes: Primer sequences are shown for 17 loci along with the length of the amplicon they would amplify in the Dothideomycete alignment. The protein sequence identity score as listed on FUNYBASE is shown. Primers shown in bold were selected for further work.

Protein sequence identity	FUNYBASE gene ID	Locus	Region length (bp)	Primer F 5'-3'	Primer R 5'-3'
35	**MS550**	***a***	**630**	**CAGACGCCTGCCGAGTTTTAT**	**CCTTCGTTGATGCGTTTAGG**
48.1	FG864	*a*	274	TGGCAGACGTGCAAAAGAAGC	GCCTCTGTCTTGTCTTGCTTCA
48.6	**MS432**	***a***	**457**	**CCAAGCTCTCTCTTTCGCGTC**	**GAGGGAGGCCATGTTCTGCTG**
26.8	MS547	*a*	518	CTTGGACAGATAAGTCTGAAAGA	GTAGCGATCAGGACAGAGGG
		b	578	GCCCGGGAAGACCATTTGCA	TTCCTCATCTTCGCCGCCTC
28.1	**MS578**	***a***	**678**	**CTGTAGAGAGTATTGAAGATC**	**AGGTGTCTTGGGGCCTTCTTG**
		b	454	TCTCAAGAAGGCCCCAAGACACC	CCAATGACGCAGGCCGCAACG
		c	578	TTGCGGCCTGCGTCATTGGGA	TCGCACAGATTGTACTCGTACTTC
		d	401	CACTTGAACGCATCTTCAACC	TAAAAAACAGGACAGTCCCTACT
44.2	MS294	*a*	496	CAAATTGACTATGCAGTGTTGCAT	AAACTCGGAAAAGATGCAGCC
		b	613	ACAGACACGTTCGGCACACCA	ACCGCCAGCCCACGACTTCT
23.7	MS393	*a*	563	ACCCGAAACTCTCCAAAGAAGA	GCTTGTTGTGCTTCACGTTGAG
		b	644	ACCTGTCAGGACCAATGTACGG	CCTAGCTTCTCGTCAAAGTTGAC
48.1	FG750	*a*	669	TCTACCAAATGACCTTGAACGG	GTTTGTTCGGCGAGTGTAAGGGT
32.4	MS374	*a*	644	ATTGGTGCATGGCACATGAGCG	GAAGCACCGACGCTCGTGGAT
		b	466	AAGGTCATTCGCGTGTGGAA	CGATGTCGTTGCGTGCCACT
40	MS320	*a*	638	AGTCACGCTACGCATCAAACTC	GGGATGAGACCAGGGAAGCC

Evolutionary relationships within the *Alternaria alternata* species group

Phylogenetic relationships were identified within the *A. alternata* species group using single and multi-locus phylogenies determined from the six genetic loci that showed the greatest number of SNPs / sequence length. Three loci were used from previous studies: Endopolygalacturonase (*endoPG*), *Alternaria* allergen1 (*Alta1*) and an Aegerolysin-like gene (*L152*). In addition, three novel FUNYBASE loci were used: *MS432*, *MS550* and *MS578*. Single locus phylogenies were constructed using 90 isolates from the University of Warwick (UoW) culture collection for each locus. A

multi-locus phylogeny was determined for 90 isolates in the UoW culture collection by concatenating sequence alignments for individual loci. DNA extraction, PCR and Sanger sequencing were performed as described in Chapter 2.

The *A. brassicicola* ex-type strain *EGS 42.002* (Simmons, 2007), was used as an out-group for phylogenetic analysis. Sequence data was generated from *A. brassicicola* (*EGS 42.002*) for each genetic locus by performing BLAST searches against the publicly available genome (in Geneious; Kearse *et al.* (2012)). Sequence data generated from the other *A. alternata* isolates (described above) were used as query sequences.

Constructing single-locus phylogenies

Bayesian phylogenies of the 90 sequences were determined for each locus. Sequence alignment was performed in Geneious (Kearse *et al.*, 2012) using the Muscle alignment algorithm. Alignments were imported to MEGA5.1 where the best fitting evolutionary model was determined using the modeltest function. The Markov chain Monte Carlo (MCMC) analysis was performed using the MrBayes plugin in Geneious. Four chains, set at a heat of 0.1 were run and were sampled every 1000 generations. Loci whose evolutionary model did not require gamma parameters were run for 10 million generations before average standard deviations were confirmed to be below 0.01. Loci whose evolutionary model required gamma parameters were run for 20 million generations before confirming standard deviations. The first 25% of trees were discarded as burn-in, after which log likelihood values were observed to be stationary. Geneious was used to calculate 50% consensus phylogenies from each MrBayes run, and was used to edit phylograms. Meaningful phylogenetic clades within phylogenies were identified by eye.

Constructing multi-locus concatenated phylogenies

Sequence alignments for genetic loci were concatenated and used to determine a multi-locus "concatenated" phylogeny. The distribution of Simmons (2007)

representative strains of *A. alternata*, *A. tenuissima*, *A. arborescens*, *A. mali* and *A. gaisen* were visually inspected in the phylogeny along with indications of host association between isolates and phylogenetic clades.

Bayesian phylogenetics was performed using the MrBayes plugin in Geneious (Kearse *et al.*, 2012). The custom command block was used to specify partitioning of the dataset by genes and to assign to each of these genes the appropriate evolutionary model. The run was performed using four heated chains, set at a heat of 0.1 and sampled every 1000 generations. Following 20 million generations average standard deviations were confirmed to be below 0.01 for the run. The first 25% of trees were discarded as burn-in, after which log likelihood values were observed to be stationary. Geneious was used to calculate 50% consensus phylogenies from each MrBayes run, and was also used to edit phylograms.

The topologies of single locus phylogenies were compared to that of the multi-locus phylogeny. The clade of the multi-locus phylogeny that each isolate was present in was indicated on the clades identified by single locus phylogenies. The consistency of the grouping of clades allowed incongruity to be identified between phylogenies.

Confirming isolates used are a representative sample of the *Alternaria alternata* species group

The 90 *Alternaria* isolates sequenced for the *L152* locus from the University of Warwick (UoW) culture collection were compared to isolates from a diverse culture collection held at the Tree Fruit Research Extension Centre (TFREC) in Wenatchee, Washington in the USA. Collaborators Dr. R.G. Roberts and S. Reymond, associated with the USDA, based at the TFREC generated *L152* sequence data for 137 isolates. Practical protocols for culture preparation in liquid medium and DNA extraction using a chloroform-phenol protocol were performed as described in Roberts *et al.* (2000). PCR and sequencing were performed using primers and annealing temperatures shown in Chapter 2 (Table 2.2).

R.G. Roberts had performed morphological identification of isolates. Morphological observations were made at 50 times magnification from growing cultures and from

spore mounts in lactic acid. Cultures had been grown on potato carrot agar (PCAgar) (recipe in Chapter 4.3) and maintained at 23 °C in a gradually drying atmosphere under a 16 hr. dark 8 hr. light cycle. Identifications were made in accordance with descriptions of Simmons (2007) (described in detail in Chapter 4.1). Sequence alignments of the 90 UoW *L152* sequences and the 137 TFREC sequences were made in Geneious (Kearse *et al.*, 2012). Bayesian phylogenetics was performed on the dataset as described for single locus alignments in 3.3.3.

3.4 RESULTS

Identifying highly variable loci

Loci that have been previously used to perform phylogenetics in the *Alternaria* genus were tested to determine variability within the *A. alternata* species group. The number of SNPs in alignments of an out-group (*A. brassicicola*) and four *A. alternata* species group taxa (*A. alternata*, *A. tenuissima*, *A. gaisen* and *A. arborescens*) were determined (Table 3.2). Alignments of rRNA loci SSU, LSU and ITS showed low variability when including *A. brassicicola* (0-15 SNPs) and no, or little variability in alignments between the *A. alternata* species group (0-1 SNPs). Alignments of commonly used gene loci (Actin, *gpd*, *TEF* and *RPB2*) showed moderate to high variability when including *A. brassicicola* (27-80 SNPs) and little variability in alignments between the *A. alternata* species group (2-5 SNPs), except for *RPB2* which showed moderate variability (23 SNPs). Alignments of uncommonly used phylogenetic loci / *A. alternata* specific gene loci (ATPase, Calmodulin, *endoPG*, *Alta1* and *L152*) showed high variability when including *A. brassicicola* (61-132 SNPs) and moderate variability in alignments between the *A. alternata* species group (20-30 SNPs) except for *L152* which showed high variability (96 SNPs).

Three highly variable loci identified from FUNYBASE were sequenced across the five representative *Alternaria* isolates to allow comparison to the loci assessed above (Table 3.2). FUNYBASE loci showed high variability when including *A. brassicicola* (103-165 SNPs) and moderate to high variability in alignments between the *A. alternata* species group (21-57 SNPs).

The percentage of variable bases in *A. alternata* species group alignments was used to identify the six most variable phylogenetic loci from those tested (*endoPG*, *Alta1*, *L152*, *MS432*, *MS578* and *MS550*; Table 3.2). Phylogenetic loci showed 0-5% polymorphic sites apart from MS550 and *L152*, which showed high numbers of SNPs per base pair length (9% and 23% respectively).

Table 3.3 Variability of 15 phylogenetic loci across *Alternaria* morphological species: Number of single nucleotide polymorphisms (SNPs) present in alignments including *A. brassicicola* or just between taxa within the *A. alternata* species group (*A. alternata*, *A. tenuissima*, *A. arborescens* and *A. gaisen*) for each locus. The six loci with the greatest % of polymorphic sites in *A. alternata* species group alignments were used for phylogenetic study.

Phylogenetic locus		Function	Length (bp)	No. SNPs including *A. brassisicola*	No. SNPs excluding *A. brassisicola*	% variable sites
rRNA loci	*SSU*	18S nuclear small ribosomal subunit	1020	0	0	0.0
	LSU	28S nuclear large ribosomal subunit	1339	7	1	0.1
	ITS	non-coding spacer regions + 5.8S nuclear small ribosomal subunit	1104	15	0	0.0
Commonly used gene loci	Actin	Actin: Cellular orginisation	939	27	2	0.2
	gpd	Glyceraldehyde 3-phosphate dehydrogenase: Glucose metabolism; oxidative stress tolerance	582	36	4	0.7
	TEF	Translation elongation factor 1: Translation of RNA	241	29	5	2.1
	RPB2	Second largest RNA polymerase subunit	865	80	23	2.7
Uncommon / *Alternaria*-specific gene loci	ATPase	Plasma membrane ATPase: Hydrogen ion transport	1209	129	30	2.5
	Calmodulin	Calcium modulated protein: Calcium binding	804	109	24	3.0
	EndoPG	EndoPolygalacturonase: Carbohydrate metabolism; cell wall degredation	464	61	20	**4.3**
	Alta1	Alternaria major allergen 1: Unknown function; involved in pathogenicity	474	66	26	**5.5**
	L152	Aegerolysin-like gene: Unknown function; involved in sporulation	425	132	96	**22.6**
Novel (FUNYBASE) loci	*MS432*	Protein of unknown function; Associates with ribosomes and has a putative RNA binding domain	433	103	21	**4.8**
	MS578	Subunit of DNA polymerase zeta	620	130	30	**4.8**
	MS550	Phosphatidylglycerolphosphate synthase	609	165	57	**9.4**

Evolutionary relationships within the *Alternaria alternata* species group

Genetic diversity was assessed within the *A. alternata* species group using single locus phylogenies determined from six highly variable genetic loci (Fig. 3.1: *a-f*): three from previous phylogenetic studies (Fig. 3.1:*a-c*) and three novel loci identified from FUNYBASE (Fig. 3.1:*d-f*). The three loci used from previous studies were Endopolygalacturonase (*endoPG*), *Alternaria* allergen1 (*Alta1)* and an Aegerolysin-like gene (*L152*). The three loci used from FUNYBASE were *MS432*, *MS550* and *MS578*. Single locus phylogenies were constructed using 90 isolates from the University of Warwick (UoW) culture collection for each locus. A phylogeny was also constructed that reflected the evolutionary history of *A. alternata* species group across multiple genetic loci from essential chromosomes (Fig.3.2). This multi-locus phylogeny was determined for 90 isolates in the UoW culture collection by concatenating sequence alignments for individual loci.

Single locus analysis of diversity within the *A. alternata* species group

Sequence data for the *endoPG* locus was ~447-448 bp in length. The multiple sequence alignment contained 24 parsimoniously informative sites between the *A. alternata* species group isolates. Nine parsimoniously informative sites coded for an amino acid substitution. No insertion/deletion (InDel) sites were present within the alignment. A Bayesian phylogeny was determined for the *endoPG* locus (Fig. 3.1:*a*). The kiruma 2-parameter model was determined to be the best model of evolution of the dataset. Nine phylogenetic clades were identified within the phylogeny (*endoPG clades: i-ix*). The position of reference isolates within the phylogeny was noted. The *A. gaisen* reference isolate and another two isolates *ex.* pear were present in a distinct, distant clade from other small-spored isolates (*endoPG: ix*). Genetic differences between the *A. gaisen* clade and the remaining isolates included three SNPs coding for amino acid substitutions. The two *A. alternata* reference isolates (*FERA 1410* and *EGS 34.016*) were present in two different *endoPG* clades (Fig. 3.1:*a*: *endoPG vi*; *vii*). The *A. mali* and *A. tenuissima* reference isolates (*EGS 38.029* and *EGS 34.015*) had the same *endoPG* haplotype and were present in the same phylogenetic clade (Fig. 3.1:*a* *endoPG v*). *A. arborescens* (*EGS 39.128*) was present in a clade with no other reference isolates (Fig. 3.1:*a*: *endoPG ii*). No reference isolates were present in five phylogenetic clades (Fig. 3.1:*a*: *endoPG i*; *iii*; *iv*; *viii*). Host associations were not observed within clades with isolates from apple, pear, strawberry and tomato distributed throughout the phylogeny and present in both *A. tenuissima* and *A. arborescens* clades.

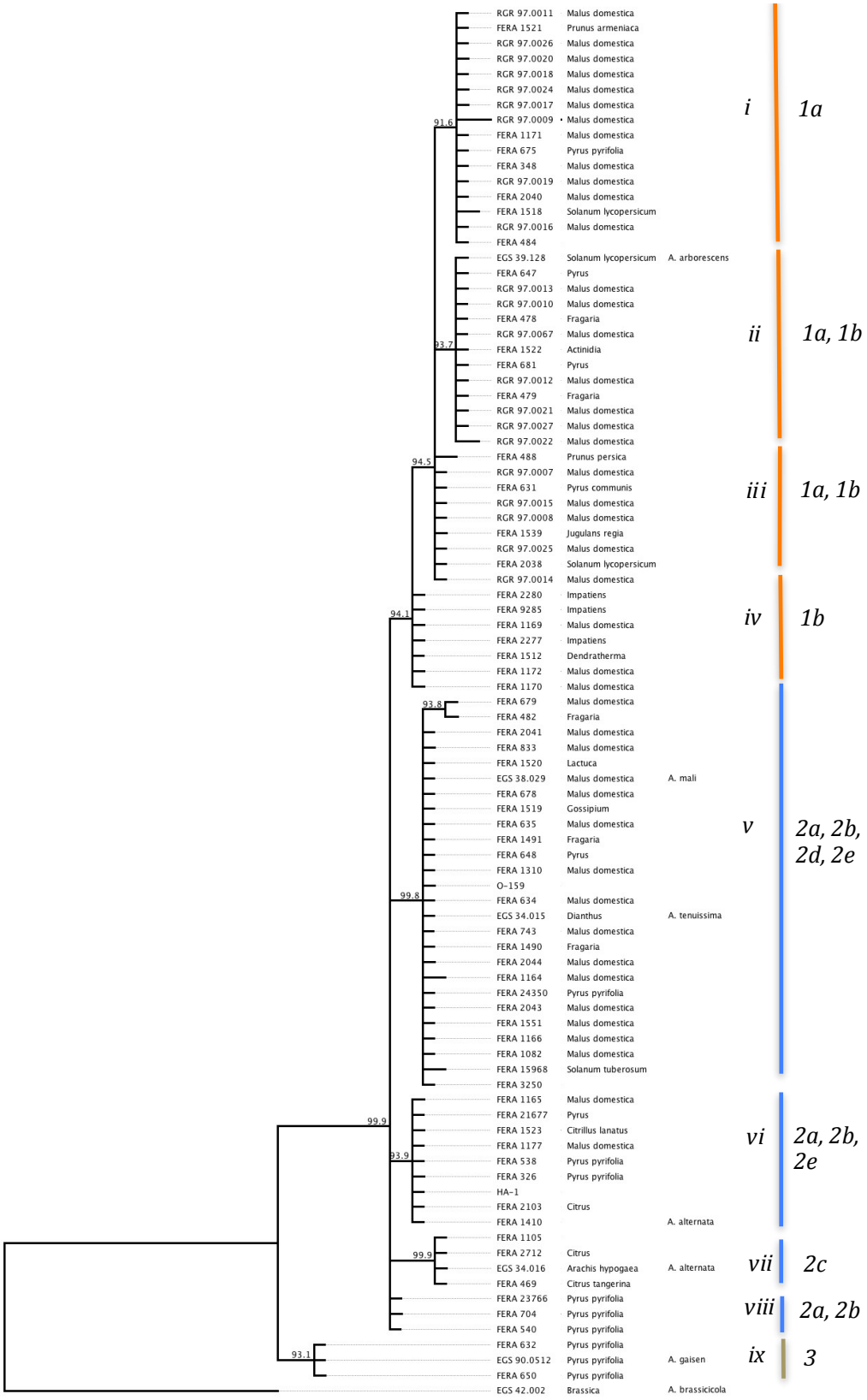


Figure 3.1.a Single locus phylogenies for 90 *Alternaria* spp. isolates (endoPG): Bayesian 50% consensus phylogeny with posterior probability shown above nodes identifying nine clades (*i-ix*). The major and minor clades on a multi-locus phylogeny (Fig. 3.2: *1a-3*) that isolates within clades *i-ix* inhabit are labelled. Host information is shown where known. *Alternaria* species names are shown for representative isolates.

Sequence data for the *Alta1* locus was 467-468 bp in length. The multiple sequence alignment contained 37 parsimoniously informative sites between the *A. alternata* species group isolates. A Bayesian phylogeny was determined for the *Alta1* locus (Fig. 3.1:*b*). Nine phylogenetic clades were identified within the phylogeny (*Alta1 clades: i-ix*). The kiruma 2-parameter model was determined to be the best model of evolution of the dataset. Position of reference isolates was noted within the phylogeny. The *A. arborescens* reference isolate (*EGS 39.128*) was present in a large clade of 35 isolates (Fig. 3.1:*b*: *Alta1 vi*). The *A. mali* reference (*EGS 38.029*) isolate was present in a clade of five isolates (Fig. 3.1:*b*: *Alta1 v*) that carried a distinct haplotype to the closely related clade containing both *A. alternata* reference isolates and the *A. tenuissima* reference isolate (*Alta1: iv; FERA 1410*, *EGS 34.016*, *EGS 34.015)*. The *A. gaisen* reference isolate (*EGS 90.0512*) was present in a distinct clade with two other isolates *ex.* pear (Fig. 3.1:*b*: *Alta1 iii)*. An InDel site was present in the DNA alignment for these three isolates. This InDel coded for a missing Thymine at base position 336 in the alignment for these isolates. No reference isolates were present in five phylogenetic clades (Fig. 3.1:*b*: *Alta1 i*; *ii*; *vi*; *vii*; *viii*). Host associations were not observed within clades with isolates *ex.* apple, pear, citrus, strawberry and tomato being distributed throughout the phylogeny.

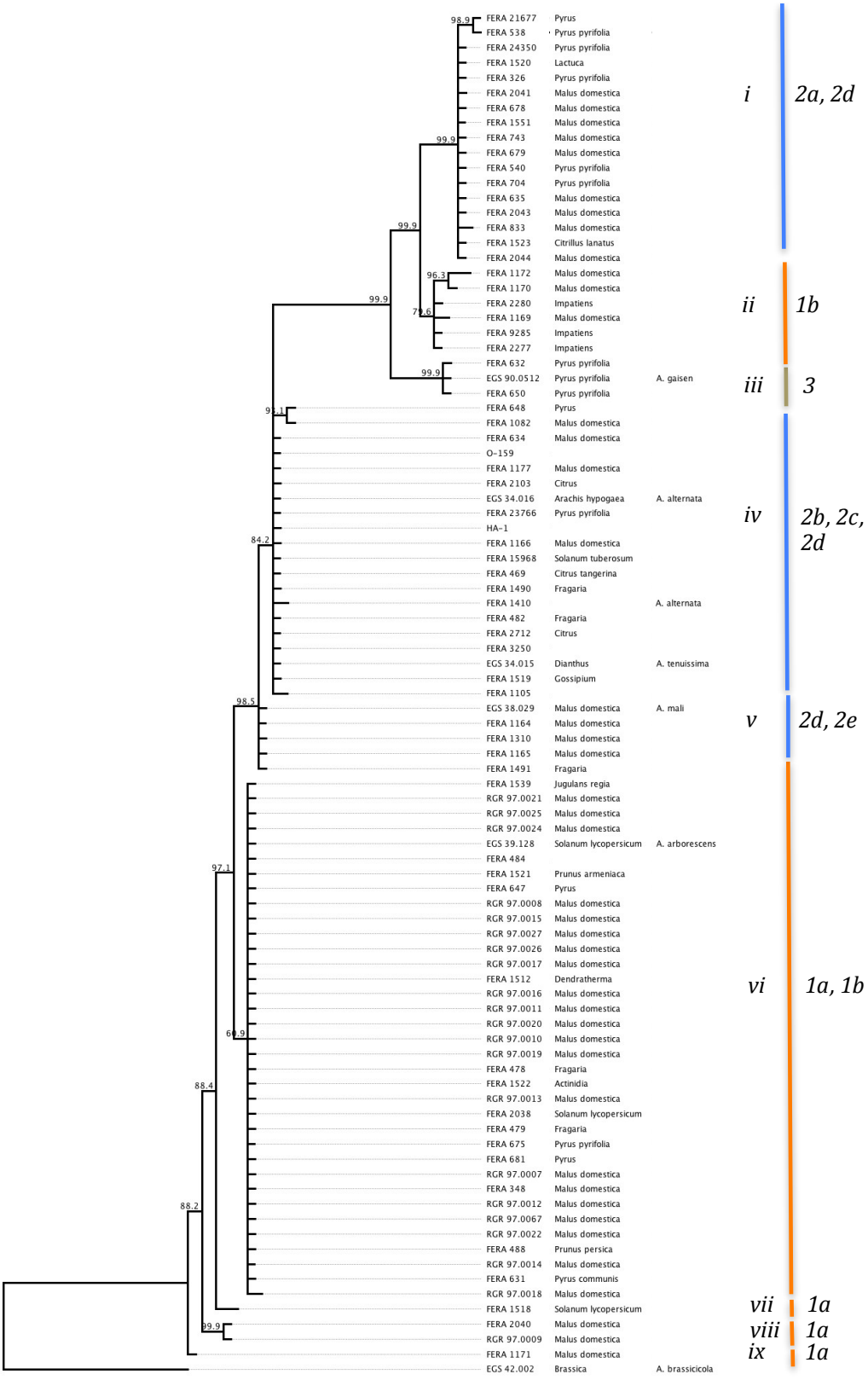


Figure 3.1b Single locus phylogenies for 90 *Alternaria* spp. isolates (Alta1): Bayesian 50% consensus phylogeny with posterior probability shown above nodes identifying nine clades (*i-ix*). The major and minor clades on a multi-locus phylogeny (Fig. 3.2: *1a-3*) that isolates within clades *i-ix* inhabit are labelled. Host information is shown where known. *Alternaria* species names are shown for representative isolates.

Sequence data for the *L152* locus was 404-414 bp in length. The multiple sequence alignment contained 121 parsimoniously informative sites between the *A. alternata* species group isolates. A Bayesian phylogeny was determined for the *L152* locus (Fig. 3.1:*c*). A kiruma 2-parameter model with a gamma coefficient of 0.59 was determined to be the best model of evolution of the dataset. Ten phylogenetic clades were identified within the phylogeny (*L152 clades: i-x*). *A. gaisen* (*EGS 90.0512*) and two other isolates *ex.* pear formed a distinct clade from other isolates in the phylogeny (Fig. 3.1:*c*: *L152 x*). An InDel site was present in these three isolates in the clade of the *A. gaisen* representative isolate. This InDel was 10 bp in length at base positions 148-148 in the sequence alignment. Five isolates were present in a clade with a similar genotype to the *A. gaisen* representative isolate but did not possess a 10 bp InDel site (Fig. 3.1:*c*: *L152: ix*). Reference isolates for *A. tenuissima* and *A. mali* (*EGS 34.015* and *EGS 38.029*) were present in the same clade (Fig. 3.1:*c*: *L152 iv*). Both *A. alternata* representative isolates (*FERA 1410* and *EGS 34.016*) were present in the same phylogenetic clade (Fig. 3.1:*c*: *L152 v*). *A. arborescens* (*EGS 39.128*) was present in a large clade of 39 isolates (Fig. 3.1:*c*: *L152 iv*). Nested within this clade was a distinct clade containing three isolates *ex.* busy lizzie (Fig. 3.1:*c*: *L152 iii*). Two other clades were nested within the *A. arborescens* clade (Fig. 3.1:*c*: *L152 i*; *ii*). In total, no reference isolates were present in five phylogenetic clades (Fig. 3.1:*c*: *L152 i*; *ii*; *iii*; *vii*; *viii*). Host associations were not observed within clades with isolates *ex.* apple, pear, citrus, strawberry and tomato being distributed throughout the phylogeny.

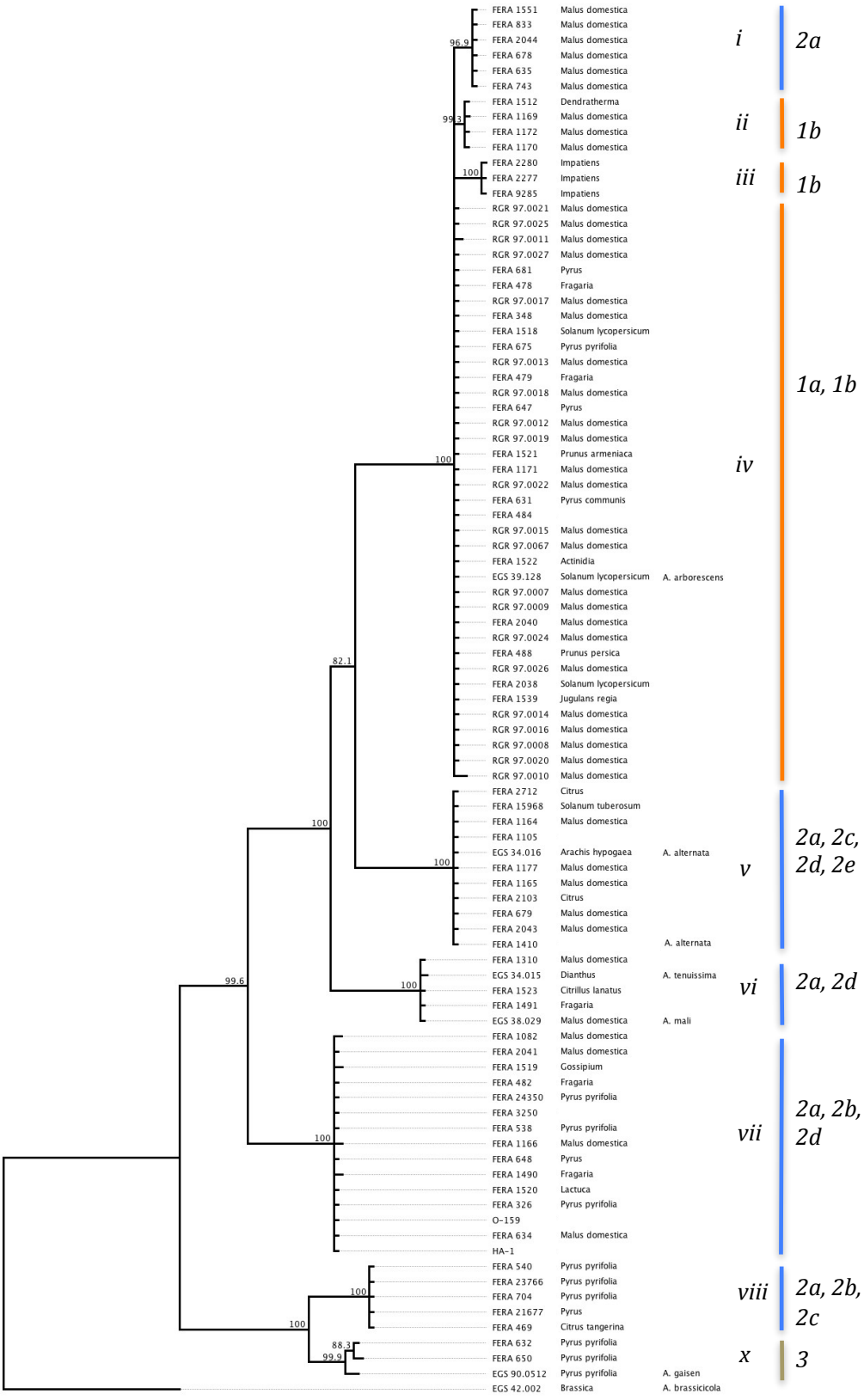


Figure 3.1c Single locus phylogenies for 90 *Alternaria* spp. isolates (L152): Bayesian 50% consensus phylogeny with posterior probability shown above nodes identifying ten clades (*i-x*). The major and minor clades on a multi-locus phylogeny (Fig. 3.2: *1a-3*) that isolates within clades *i-x* inhabit are labelled. Host information is shown where known. *Alternaria* species names are shown for representative isolates.

The sequence alignment for the *MS432* locus was 428-429 bp in length. The multiple sequence alignment contained 26 parsimoniously informative sites between the *A. alternata* species group isolates. An InDel site was present for two isolates (634 and 15968) which were otherwise the same haplotype as the *A. alternata*, *A. tenuissima* and *A. mali* representative isolates. This InDel was at base position 35 in the alignment, encoding an adenine in all other isolates. A Bayesian phylogeny was determined for the *MS432* locus (Fig. 3.1:*d*). Six phylogenetic clades were identified within the phylogeny (*MS432 clades: i-vi*). The kiruma 2-parameter model with a gamma parameter was determined to be the best model of evolution of the dataset. *A. mali*, *A. tenuissima* and *A. alternata* reference isolates (*EGS 38.029*, *EGS 34.015, FERA 1410* and *EGS 34.016*) shared a common haplotype and were present in a large clade of 42 isolates (Fig. 3.1:*d*: *MS432 iv*). The reference isolate for *A. gaisen* (*EGS 90.0512*) was positioned in a distinct clade along with two other isolates *ex.* pear (Fig. 3.1:*d*: *MS432 v*). The *A. arborescens* reference isolate (*EGS 39.128*) was present in a clade of 29 isolates (Fig. 3.1:*d*: *MS432 i*). No reference isolates were present in three phylogenetic clades (Fig. 3.1:*d*: *MS432 ii*; *iii*; *iv*). In general, host associations were not observed within clades with isolates *ex.* apple, pear, citrus, strawberry and tomato being distributed throughout the phylogeny. Three isolates *ex.* Impatiens (syn. busy lizzie) possessed a distinct haplotype (Fig. 3.1:*d*: *MS432 iii*).

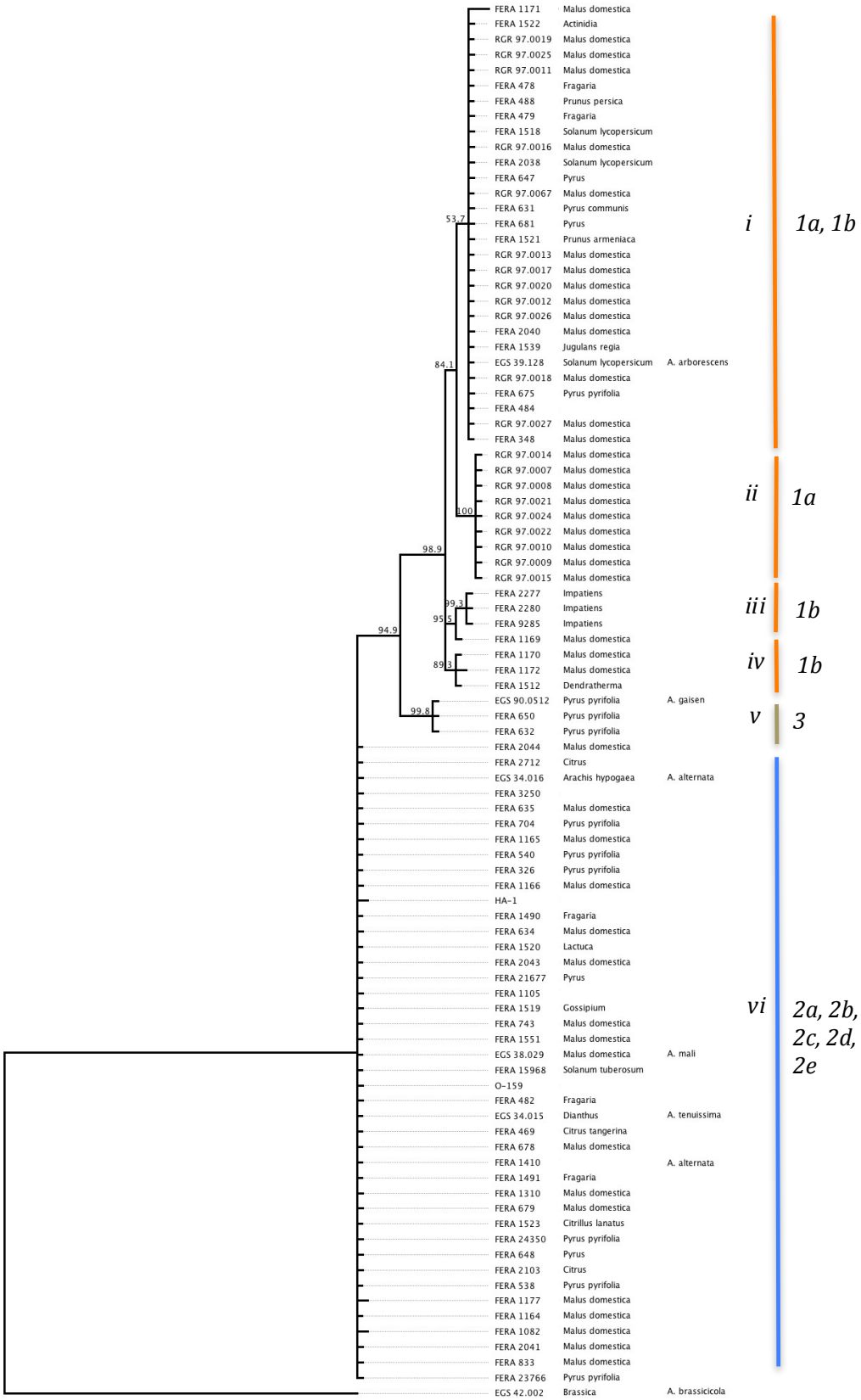


Figure 3.1d Single locus phylogenies for 90 *Alternaria* spp. isolates (MS432): Bayesian 50% consensus phylogeny with posterior probability shown above nodes identifying six clades (*i-vi*). The major and minor clades on a multi-locus phylogeny (Fig. 3.2: *1a-3*) that isolates within clades *i*-v*i* inhabit are labelled. Host information is shown where known. *Alternaria* species names are shown for representative isolates.

Sequence data for the *MS550* locus was 597 bp in length. The multiple sequence alignment contained 99 parsimoniously informative sites between the *A. alternata* species group isolates. Two InDel sites were present between isolates in the alignment. A Bayesian phylogeny was determined for the *MS550* locus (Fig. 3.1:*e*). Nine phylogenetic clades were identified within the phylogeny (*MS550 clades: i-ix*). A Tamura-Nei model with a gamma coefficient of 0.63 was determined to be the best model of evolution for the dataset. The *A. arborescens* reference isolate (*EGS 39.128*) was placed in a distinct phylogenetic clade of six isolates (Fig. 3.1:*e*: *MS550 vii*). The *A. mali*, *A. tenuissima* and one of the *A. alternata* reference isolates (*EGS 38.029*, *EGS 34.015* and *FERA 1410*) were present in a single phylogenetic clade (Fig. 3.1:*e*: *MS550 vi*). The second *A. alternata* isolate (*EGS 34.016*) was clustered within this clade and carried a closely related haplotype (Fig. 3.1:*e*: *MS550 v*). Two InDel sites in the alignment supported the distinction of the clade containing *A. alternata*, *A. tenuissima* and *A. mali* representative isolates. The first was at base position 57 in the alignment where a cytosine was present in all of the isolates in this clade. The second InDel was at base position 116 and encoded for a cytosine in all isolates not part members of this clade. The *A. gaisen* reference isolate (*EGS 90.0512*) was present in a distinct clade along with two other isolates *ex.* pear (Fig. 3.1:*e*: *MS550 iii*). No reference isolates were present in five phylogenetic clades (Fig. 3.1:*e*: *MS550: i*; *ii*; *iv*; *viii*; *ix*). Host associations were not observed within clades with isolates *ex.* apple, pear, citrus, strawberry and tomato being distributed throughout the phylogeny.

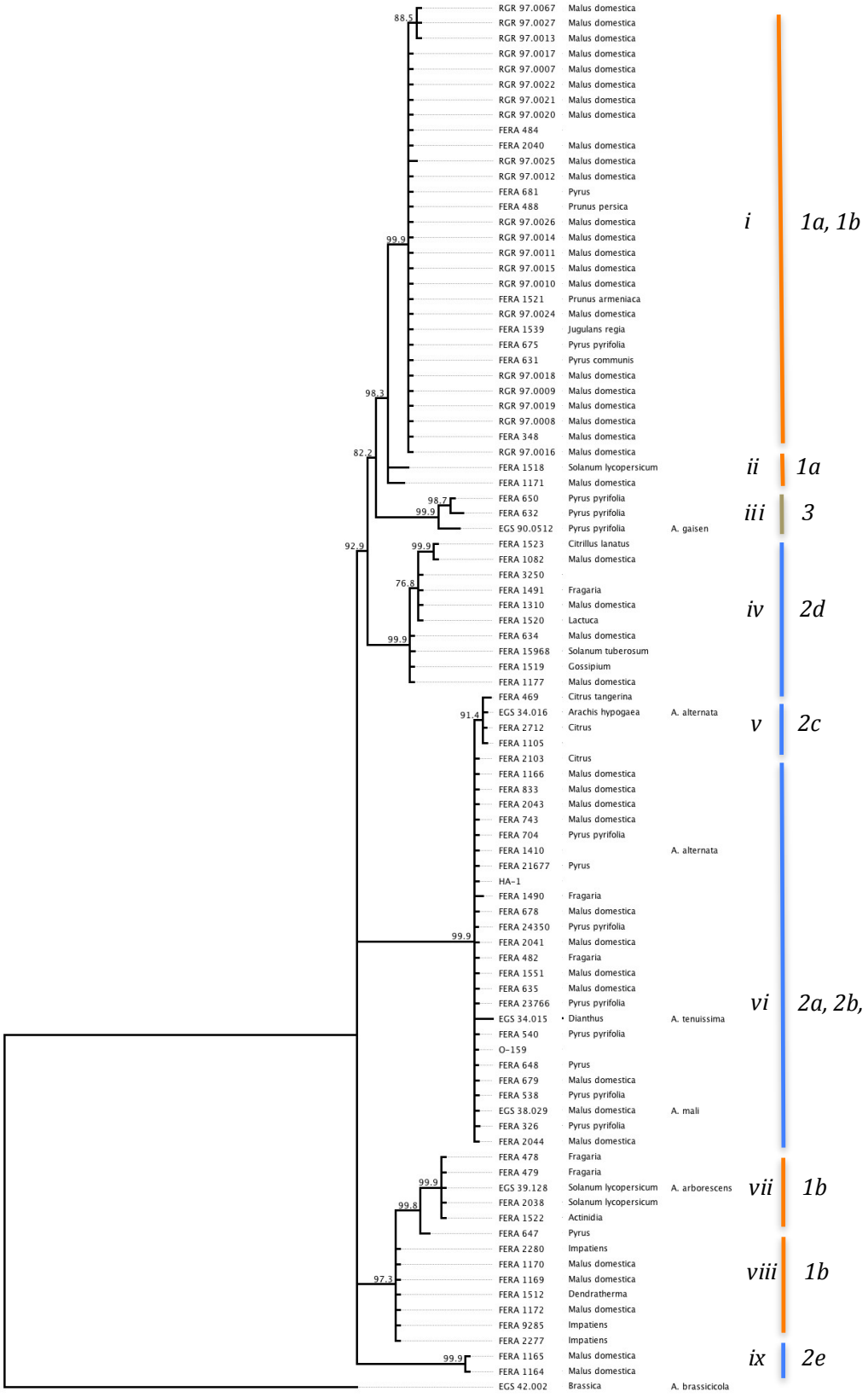


Figure 3.1e Single locus phylogenies for 90 *Alternaria* spp. isolates (*MS550*): Bayesian 50% consensus phylogeny with posterior probability shown above nodes identifying nine clades (*i-ix*). The major and minor clades on a multi-locus phylogeny (Fig. 3.2: *1a-3*) that isolates within clades *i-ix* inhabit are labelled. Host information is shown where known. *Alternaria* species names are shown for representative isolates.

Sequence data for the *MS578* locus was 620 bp in length. The multiple sequence alignment contained 42 parsimoniously informative sites between the *A. alternata* species group isolates. No InDel sites were present between isolates in the alignment. A Bayesian phylogeny was determined for the *MS578* locus (Fig. 3.1:*f*). 11 phylogenetic clades were identified within the phylogeny (*MS578* clades: *i*-*xi*). The Kimura 2-parameter mode with a gamma parameter was determined to be the best model of evolution for the dataset. *A. gaisen* (*EGS 90.0512*) was placed in a distant, distinct clade with two other isolates *ex.* pear (Fig. 3.1:*f*: *MS578 xi*). One of the two *A. alternata* reference isolates, the *A. mali* and the *A. tenuissima* reference isolates (*FERA 1410* and *EGS 38.029* and *EGS 34.015*) shared similar haplotypes and were present in a large clade of 38 isolates (Fig. 3.1:*f*: *MS578*). The second *A. alternata* reference isolate (*EGS 34.016*) was placed in a distinct clade along with two citrus isolates (Fig. 3.1:*f*: *MS578 viii*). The *A. arborescens* reference isolate (*EGS 39.128*) was present in a distinct clade not shared with other reference isolates (Fig. 3.1:*f*: *MS578 v*). No reference isolates were present in seven phylogenetic clades (Fig. 3.1:*f*: *MS578 i*; *ii*; *iii*; *iv*; *vi*; *vii*; *ix*). Host associations were not observed within clades with isolates *ex.* apple, pear, citrus, strawberry and tomato being distributed throughout the phylogeny.

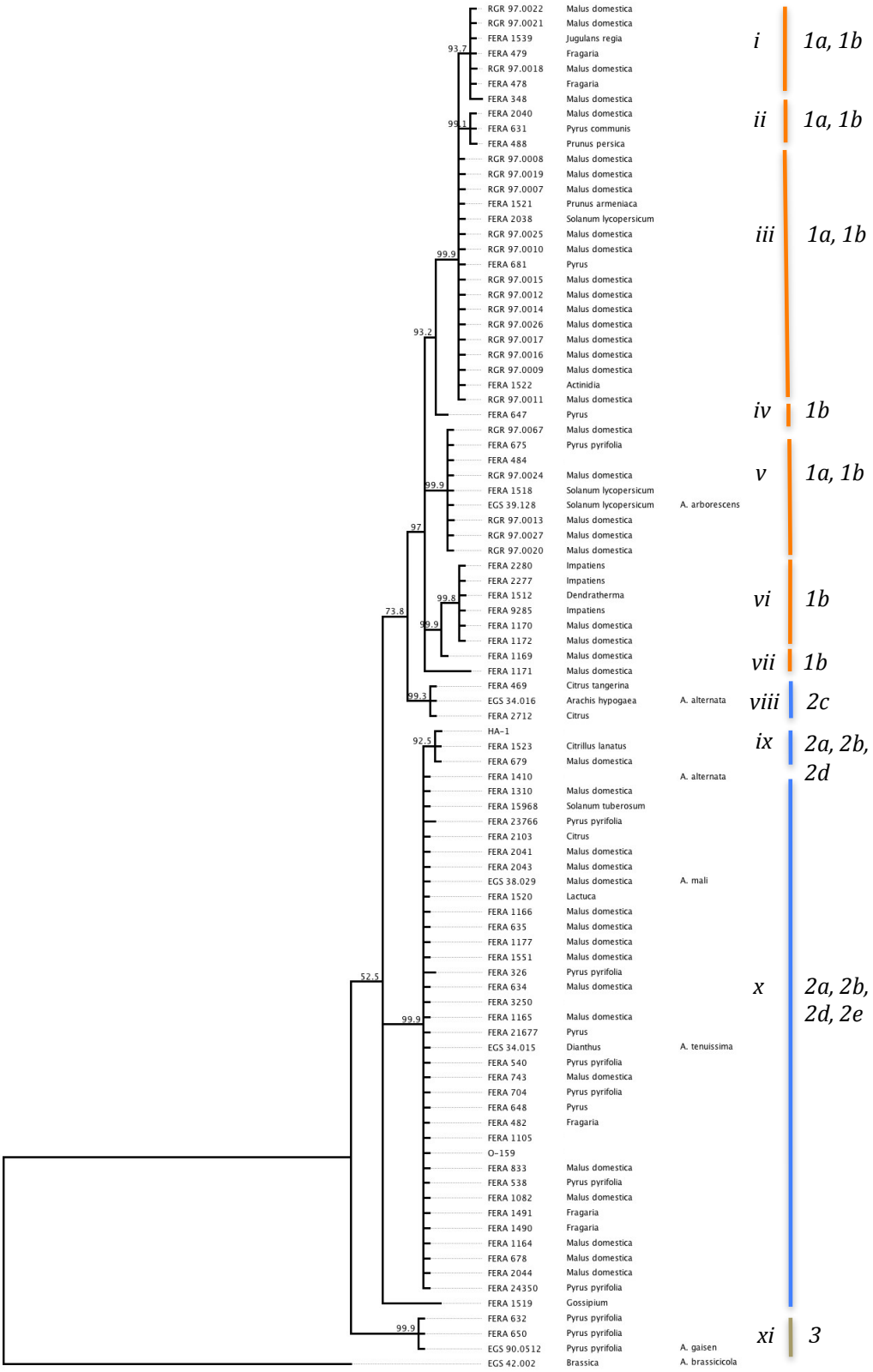


Figure 3.1f Single locus phylogenies for 90 *Alternaria* spp. isolates (MS578): Bayesian 50% consensus phylogeny with posterior probability shown above nodes identifying nine clades (*i-ix*). The major and minor clades on a multi-locus phylogeny (Fig. 3.2: *1a-3*) that isolates within clades *i-ix* inhabit are labelled. Host information is shown where known. *Alternaria* species names are shown for representative isolates.

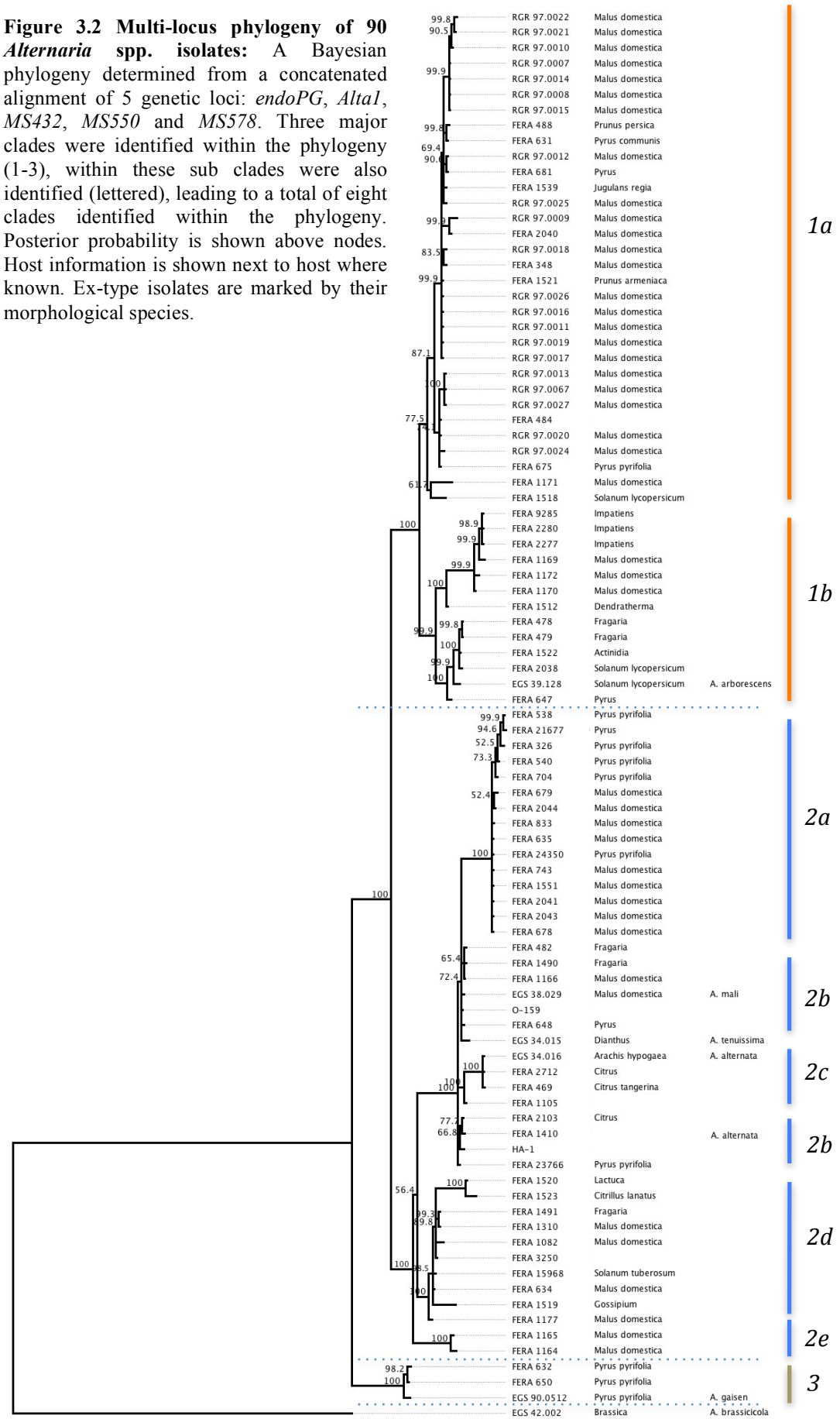
Multi-locus analysis of diversity within the *A. alternata* species group

A six-gene concatenated alignment was made from *endoPG*, *Alta1*, *L152*, *MS432*, *MS550* and *MS578* loci and a Bayesian phylogenetic tree was constructed. However, Bayesian posterior probability did not decrease to below 0.01 when the *L152* locus was included in the concatenated alignment. As a result the *L152* locus was removed from the alignment.

A five-gene concatenated alignment was made from *endoPG*, *Alta1*, *MS432*, *MS550* and *MS578* loci. The concatenated alignment was 2,578bp in length and contained 237 parsimoniously informative sites between isolates. A Bayesian phylogeny was determined from the alignment (Fig. 3.2). Three major clades were identified within the phylogeny (Clades 1-3) and minor clades (sub-clades) were identified within these. This led to a total identification of eight clades within the phylogeny (Clades 1*a*-1*b*, 2*a*-2*e* and 3).

Phylogenetic Clade 3 represented the basally-placed distinct clade consisting of *A. gaisen* (*EGS 90.0512*) and two other isolates *ex.* pear. Due to this clade being distinct from other *Alternaria* isolates and containing only pear isolates this clade is considered to represent the taxon *A. gaisen*. The second phylogenetic clade consisted of isolates from a diverse range of hosts. It also included the reference isolates for *A. mali*, *A. tenuissima* and A. *alternata* (*EGS 38.029*, *EGS 34.015, FERA 1410* and *EGS 34.016*). Reference isolates were present in sub-clades 2*b* and 2*c*. Sub-clades 2*a*, 2*d* and 2*e* did not show bias towards a single host, containing isolates from multiple hosts in all clades except Clade 2*e*, which consisted of only 2 isolates. Clade 2 was considered to represent *A. alternata*, *A. tenuissima* and *A. mali* as a single taxon due to representative isolates showing genetic similarity over the five loci. Phylogenetic Clade 1 consisted of two sub-clades. Clade 1*b* contained the *A. arborescens* representative isolate (*EGS 39.128*). The variability within phylogenetic Clade 2 was considered to represent population level variation within a single taxon, genetic differences between Clade 1*a* and *1b* were considered to represent population level variation within the taxon *A. arborescens*. Clade 1 contained isolates from a diversity of hosts. Isolates from tomato strawberry and impatiens were noted to share similar genotypes within each host, being positioned close together.

Figure 3.2 Multi-locus phylogeny of 90 *Alternaria* spp. isolates: A Bayesian phylogeny determined from a concatenated alignment of 5 genetic loci: *endoPG*, *Alta1*, *MS432*, *MS550* and *MS578*. Three major clades were identified within the phylogeny (1-3), within these sub clades were also identified (lettered), leading to a total of eight clades identified within the phylogeny. Posterior probability is shown above nodes. Host information is shown next to host where known. Ex-type isolates are marked by their morphological species.



Comparison of single and multi-locus phylogenies

Congruence was assessed between the clades identified in a five-gene multi-locus phylogeny and single locus phylogenies (Fig. 3.1:*a-f*). The separation of the minor Clades 1*a* and 1*b* was not concordant through all single locus phylogenies. Incongruity was present in the *endoPG* and *L152* phylogenies (Fig. 3.1:*a*, *c*). The separation of the minor Clades 2*a*, 2*b*, 2*c*, 2*d* and 2*e* was not concordant through all single locus phylogenies. Incongruity was present between *endoPG*, *Alta1*, *L152* and *MS578* (Fig. 3.1: *a-c*, *f*). Concordance was observed between major phylogenetic Clades 1, 2 and 3 across all single locus phylogenies (Fig. 3.1:*a-f*). Principles of GSR support the separation of designation of these three clades as distinct lineages (Fig. 3.3).

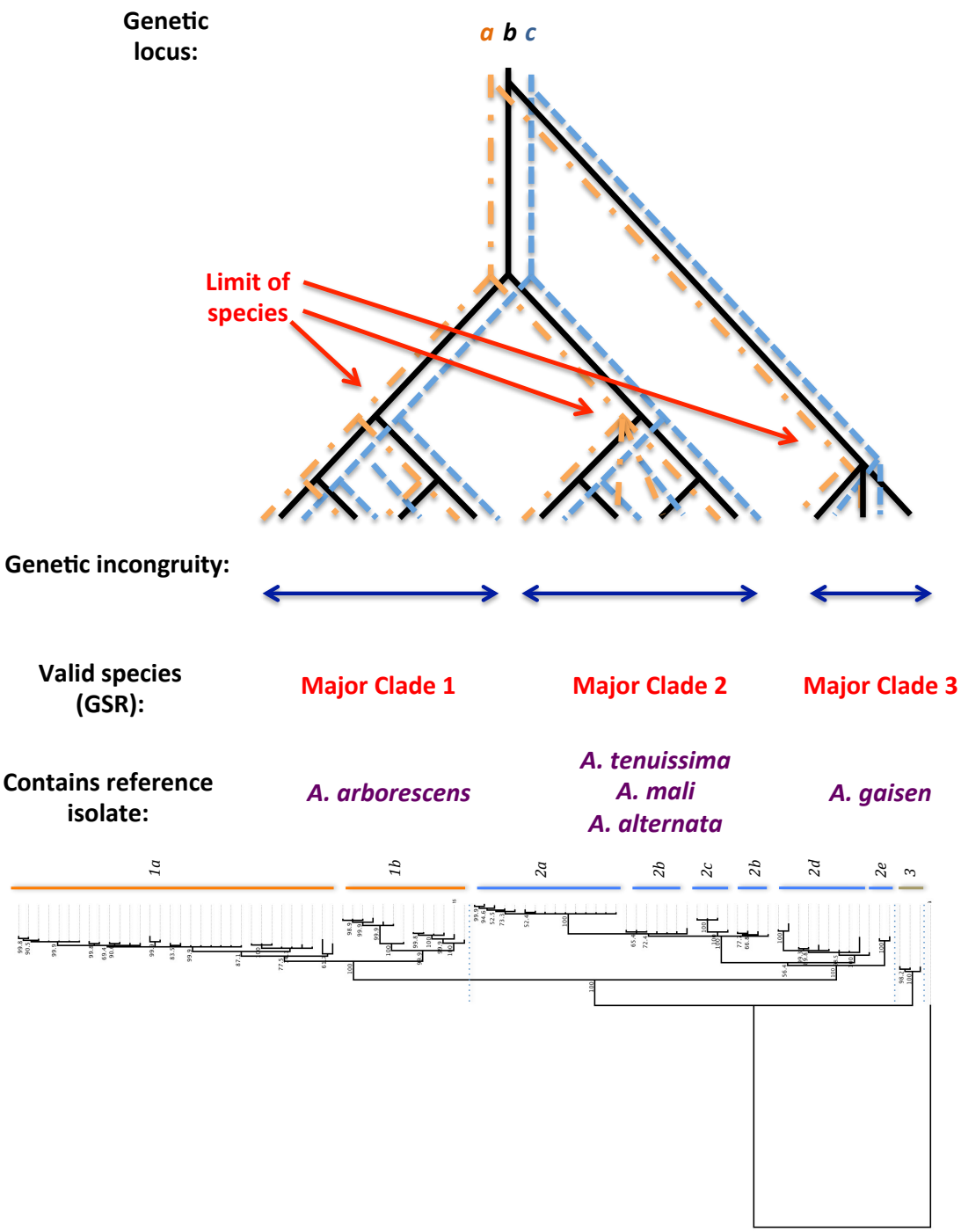


Figure 3.3 Species recognition by genealogical concordance identifies three lineages in *Alternaria alternata*: Diagram shown in three parts, the top part demonstrates principles of GSR showing concordance between species and incongruity within species for three hypothetical loci (*a*, *b*, *c*). The middle part shows the application of these principles to major clades identified in the *A. alternata* species group multi-locus phylogeny. The bottom part of the diagram shows an edited image of the multi-locus phylogeny including identification of congruent major clades (numbered) and incongruent minor clades (lettered).

Comparing *L152* genotypes between culture collections

To ensure that the genetic diversity of the UoW culture collection reflected the genetic diversity of other *A. alternata* species group culture collections the genetic diversity of the University of Warwick (UoW) culture collection across *L152* was compared to a diverse set of isolates from the Tree Fruit Research Extension Centre (TFREC). A sequence alignment of 420 bp was generated for the combined UoW and TFREC dataset. The multiple sequence alignment contained 201 parsimoniously informative sites between the small-spored isolates. A HKY model with a gamma parameter was used as the evolutionary model for the dataset. A Bayesian phylogeny showed that two clades were presented in the TFREC culture collection that did not contain isolates from the UoW culture collection (Fig. 3.4: *a*-*b*). One of these clades contained three isolates considered representative of *A. turkisafria* and was indicated to be closely related to a clade containing the *A. mali* (*EGS 38.029*) and *A. tenuissima* (*EGS 34.015*) representative isolate. The second clade consisted of three isolates recorded as representing the morphological species *A. tabasco*, one isolate representing *A. novea* and two isolates representing *A. limicola*.

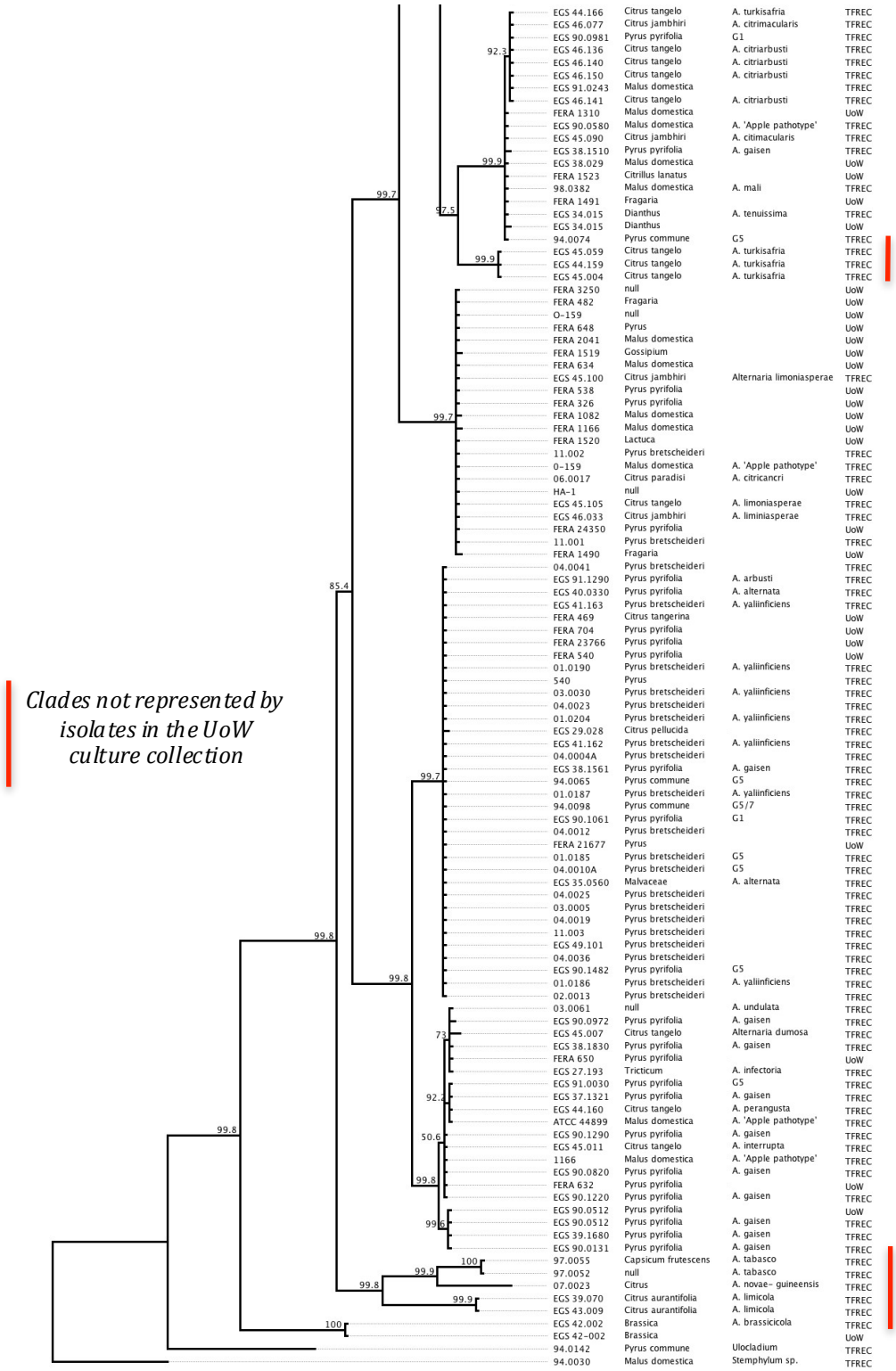
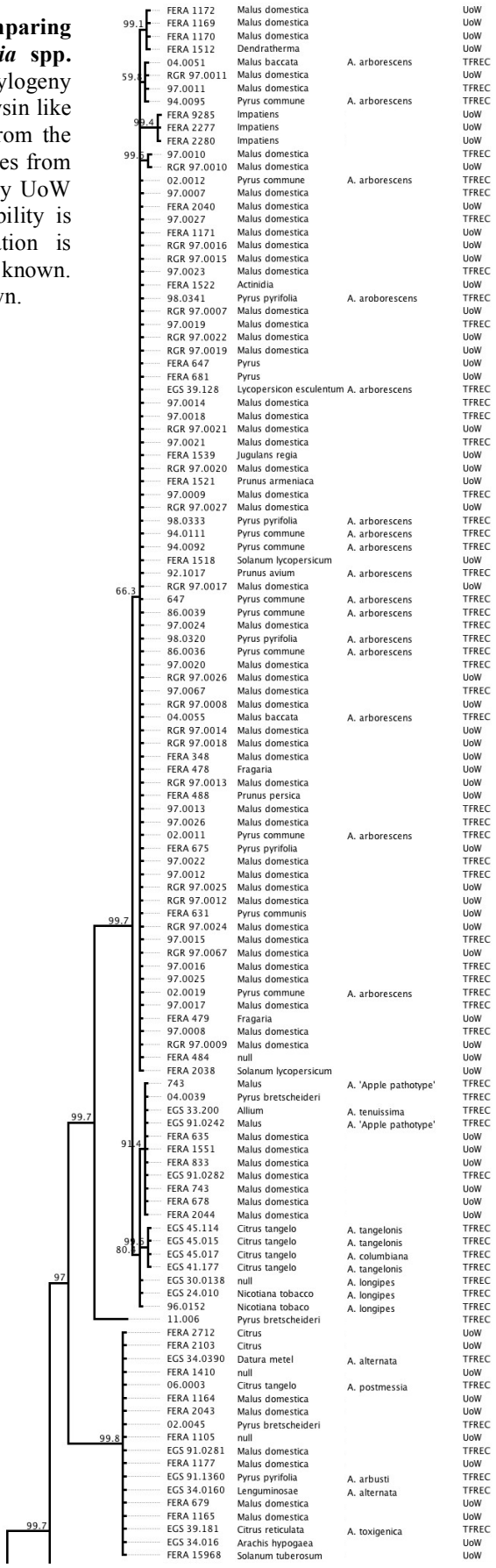


Figure 3.4.*a* *L152* phylogeny comparing genetic diversity between *Alternaria* spp. culture collections: A Bayesian phylogeny for the *L152* locus (part of an aegerolysin like gene) sequenced across 90 isolates from the UoW culture collection and 170 isolates from the TFREC. Clades not represented by UoW isolates are marked. Posterior probability is shown above nodes. Host information is shown next to host where known. Morphological identifications are shown.

Figure 3.4.*b* *L152* phylogeny comparing genetic diversity between *Alternaria* spp. culture collections: A Bayesian phylogeny for the *L152* locus (part of an aegerolysin like gene) sequenced across 90 isolates from the UoW culture collection and 170 isolates from the TFREC. Clades not represented by UoW isolates are marked. Posterior probability is shown above nodes. Host information is shown next to host where known. Morphological identifications are shown.



3.5 DISCUSSION

Genetic diversity in the isolates used was representative of *Alternaria alternata*

The UoW culture collection showed comparable genetic diversity within the *A. alternata* species group to the diverse set of isolates in the TFREC culture collection (Fig 3.8). Two clades were present in the TFREC culture collection that were not represented in the UoW culture collection. One of these represented isolates of *A. limicola* and *A. tabasco*. *A. limicola* has been shown to be a member of the *Alternaria* section *porri*, rather than the *A. alternata* species group by Lawrence *et al.* (2013), as shown in Figure 1.2. The second of these represented three isolates that were closely related to the *A. tenuissima* and *A. mali* representative isolates. As this was the only haplotype absent from the UoW culture collection and it showed close relationship to other clades the culture collection was considered representative.

Genealogical concordance identifies three subspecies in *Alternaria alternata*

One study has previously been performed to resolve the *A. alternata* species group (Andrew *et al.*, 2009). Apart from this other studies have focussed on particular pathosystems within the species group such as citrus (Peever *et al.*, 2004), pistachio (Pryor and Michailides, 2002), apple dry core rot (Kang *et al.*, 2002) or on the production of HSTs (Kusaba and Tsuge, 1995b). These studies have increased our knowledge of these individual pathosystems but do not increase our understanding of the *A. alternata* species group. This representative collection has been used to investigate evolutionary relationships within the *A. alternata* species group, performing multi-locus phylogenetics using five highly variable loci. From this three major lineages have been identified within the species group. It is recommended that these be considered as three sub-species of *A. alternata*.

The three major phylogenetic clades (Fig. 3.2: Clades 1-3) were congruent across each of the individual gene phylogenies (Fig. 3.1:*a-f*) whereas incongruence was

observed between minor phylogenetic clades (1*a*-1*b*; 2*a*-2*e*). Principles of genealogical concordance species recognition (GSR) are based upon identifying congruence and incongruity between tree topologies when performing multi-locus phylogenetics (Taylor *et al.*, 2000). Principles of GSR have been applied in taxonomically complex groups, from lichenised fungi in the *Cladia aggregata* complex (Parnmen *et al.*, 2012) to the identification of species complexes within plant pathogens such as *Fusarium graminearum* (O'Donnell *et al.*, 2004), and the Dothideomycete pathogen *Phaeospharea nodorum* (McDonald *et al.*, 2012). GSR is applied to sexual fungi as movement from congruence to incongruity denotes gene flow within species (Rintoul *et al.*, 2012). Evidence that recombination has occurred within the *A. alternata* species group is presented in Chapter 6 and supports the use of GSR. Concordance is shown within the lineages of the three major phylogenetic clades supporting these as distinct lineages, whereas incongruity is shown within minor phylogenetic clades (Fig. 3.3). Taxonomic re-evaluation of the *A. alternata* species group is discussed below and in Chapter 4 (section 4.5).

Support for *Alternaria alternata* subspecies

This is the first time GSR concepts have been used to identify genetically distinct sub-species within the *A. alternata* species group. Molecular data from a number of previous studies in particular pathosystems support the identification of three sub-species and are discussed below. Further support from morphological studies is presented in Chapter 4.

Phylogenetics was performed on the *A. alternata* species group by Andrew *et al.* (2009). Multi-locus sequencing was performed but phylogenies were presented as single gene phylogenies rather than concatenating data to make a multi-locus phylogeny. Their neighbour joining *endoPG* phylogeny identified four phylogenetic clades within the *A. alternata* species group, fewer than the ten identified in (Fig. 3.1:*a*). This was due to bootstrap resampling of neighbour joining phylogenies showing low support for some nodes, and therefore fewer clades could be identified with confidence. This is in contrast to this study where all clades were well supported (posterior probability was greater than 90%). Bayesian posterior probability has been

reported to be more prone to over supporting clades in phylogenies than bootstrapping methods (as performed on a neighbour joining phylogeny) (Alfaro *et al.*, 2003), so direct comparisons between these statistics may be misleading. Reference isolates used in this study were also used in Andrew *et al.* (2009). The placement of these reference isolates shows an agreement of tree topologies between Figure 3.1 and the results of Andrew *et al.* (2009) (Fig. 3.5). Both of these phylogenies show *A. alternata* as being present in a separate clade, a finding that was not supported by multi-locus phylogenetics in this study (Fig. 3.2).

A. gaisen (*EGS 90.0512*) has been used as an out-group when performing phylogenetics on *Alternaria* isolates causing disease in citrus (Akimitsu *et al.*, 2003, Peever *et al.*, 2004). All isolates sharing this *endoPG* haplotype to date have been isolated from *Pyrus pyrifolia* (Asian pear) Andrew *et al.* (2009) (Fig. 3.5). This may indicate some level of host specificity in this lineage.

Figure 3.5 *Alternaria* spp. endopolygalacturonase phylogeny from Andrew *et al.* (2009): Neighbour joining phylogeny of 141 *Alternaria* spp. from a range of hosts (shapes) resolved into six clades (1-6). Bold branches mark bootstrap support over 70 %. Reference isolates of *A. alternata* (*EGS 34.016*), *A. tenuissima* (*EGS 34.029*), *A. arborescens* (*39.128*) and *A. gaisen* (*EGS 90.0152*) were used in phylogeny shown and in Fig. 3.1:*a*. Their position is marked along with their *endoPG* clade in Fig. 3.1:*a*. Image adapted.

Species identification by GSR uses congruence in tree topology across multiple genetic loci to show genetic isolation of species (Taylor *et al.*, 1999). Where incongruence is present then genetic flow may be occurring and population level variation is being observed. Molecular techniques that are based upon random amplification of DNA across an isolate's genome (Xu, 2006) such as random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP), can also be considered to work in the same manner as GSR, as clades will

only be supported at the species level (where polymorphic cites are congruent within species). Phylogenetic studies using these techniques offer support for identification of major phylogenetic clades as discrete lineages.

A worldwide study of citrus pathogens used RAPD as well as *endoPG* sequence data to resolve lineages (Peever *et al.*, 2002). Clades were not identified within a dendrogram calculated from RAPD profiles, but it was noted that distant genotypes could be present in the same geographic location. The RAPD profile presented in by Peever *et al.* (2002) is observed to separate isolates into two major phylogenetic clades (Fig. 3.6). This may support the identification of discrete phylogenetic lineages (Clades 1 and 2) in this study. Phylogenetic Clade 3 was shown to be distinct from these isolates through it being used as an out-group in the *endoPG* phylogeny of the same study (Peever *et al.*, 2002),

Identification of three major clades supports the current species descriptions of *A. arborescens* (phylogenetic Clade 1) and *A. gaisen* (phylogenetic Clade 3) as distinct sub-species. However the identification of another lineage (phylogenetic Clade 2) does not support the taxonomic framework of *A. tenuissima*, *A. mali* and *A. alternata* as distinct species as are presented in Simmons (2007). The *A. mali* reference isolate (*EGS 38.029*) was found to have the same haplotype as the *A. tenuissima* reference isolate (*EGS 34.015*) in many of the individual loci phylogenies. This indicates that *A. mali* is not a distinct taxon and that it is in fact a re-description of an *A. tenuissima* isolate that was causing disease on apple. The ex-type *A. alternata* representative isolate (*EGS 34.016*) was placed in a separate phylogenetic clade from other reference isolates in *endoPG* and *MS578* phylogenies but was closely related in the phylogenies other loci. Considering these results, there is no support for *A. alternata* as a distinct genetic taxon from *A. tenuissima* or *A. mali*.

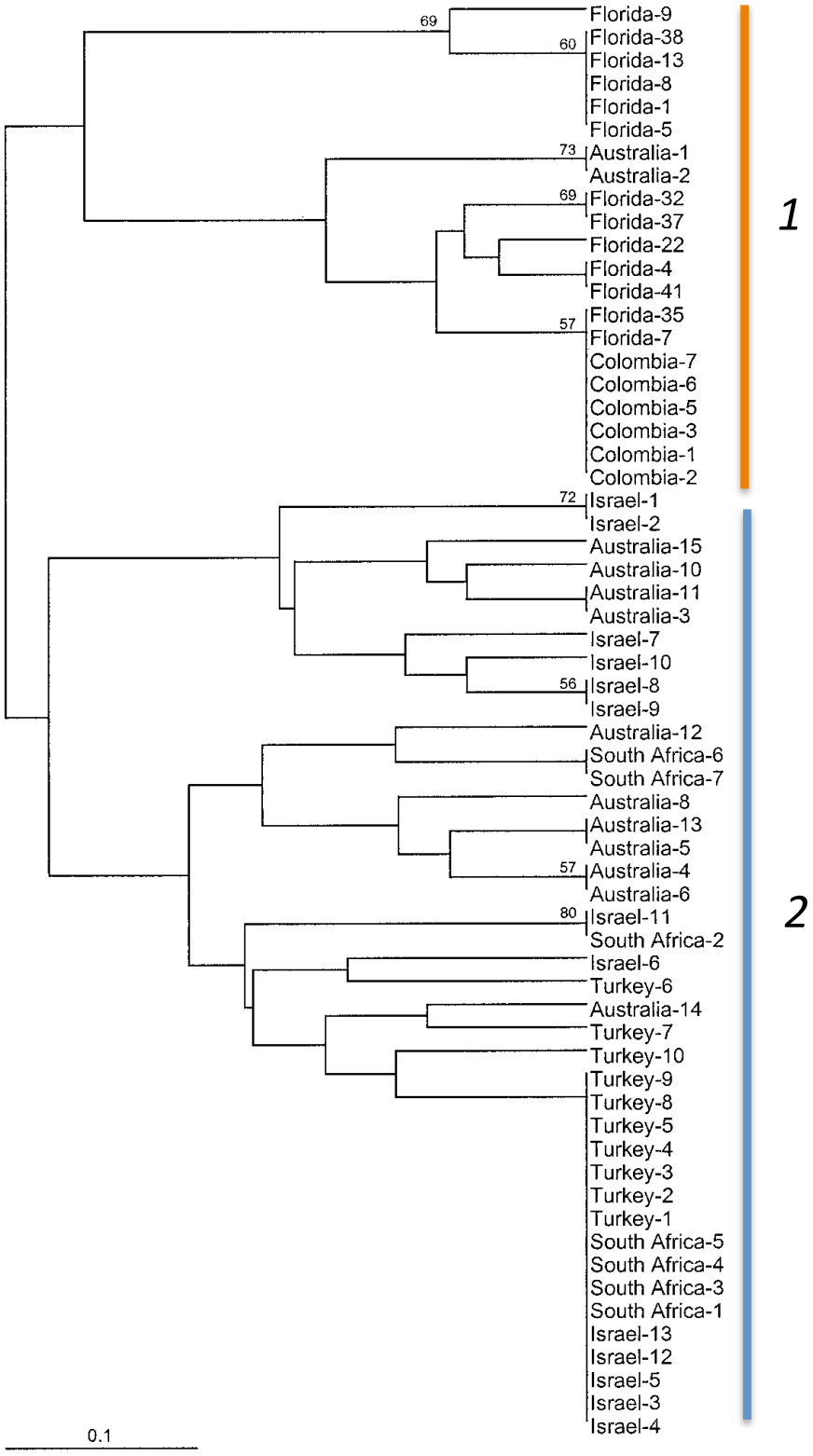


Figure 3.6 Phylogeny of RAPD profiles for a worldwide collection of *Alternaria* spp. isolates *ex. Citrus*: Phylogeny estimated from random amplified polymorphic DNA (RAPD) profiles for a worldwide sample of *Citrus* isolates. Phylogeny calculated using clustering distance matrix (Jackard coefficient). Image adapted from (Peever *et al.*, 2002), modified by identification of two phylogenetic clades. These may relate to phylogenetic Clades 1 and 2 (Fig. 3.2).

Identifying highly variable loci

Loci for which primers had been specifically designed for phylogenetics within the *Alternaria* genus showed highest levels of variability (2-23% polymorphic sites). This shows the advantage of designing loci specifically for phylogenetics within a system rather than using primers with broad specificity. Recently published ATPase and Calmodulin loci showed moderate numbers of SNPs in *A. alternata* species group alignments (24 and 30 SNPs) but due to their length they showed lower variability per base (2% and 3%) than *endoPG* (4%), *Alta1* (5%) and *L152* (23%). As such *endoPG*, *Alta1* and *L152* loci were considered most suitable for further analysis.

The *endoPG* locus has been used to resolve small-spored *Alternaria* in a number of studies. It has previously been used to show variation of genotypes in the citrus pathosystem (Timmer *et al.*, 2003, Peever *et al.*, 2005). It has also previously been used to test resolution across the entire *A. alternata* species group but was considered inadequate to resolve morphological species within the small-spored *Alternaria* (Andrew *et al.*, 2009).

endoPG primers, designed in Peever *et al.* (2004), have only been used within the *A. alternata* species group and it is unknown whether they could be used on a wider phylogenetic context in the *Alternaria* genus. The *Alta1* locus primers have been used in previous studies to perform resolve evolutionary relationships within the *Alternaria* genus and closely related *Embellisia* and *Ulocladium* (Lawrence *et al.*, 2011, Lawrence *et al.*, 2013). As such the *Alta1* locus is an appropriate locus for performing genus wide phylogenetic studies within the *Alternaria*.

The *L152* locus showed the greatest level of variability within the small-spored *Alternaria* (132 SNPs, leading to 23% of sites being polymorphic). The function of Aegerolysins is still not fully understood (Berne *et al.*, 2009), but this variability suggests that similar genes could be considered for phylogenetics on other closely related species groups. *L152* has previously been reported to show variation between *Alternaria* isolates but the dataset which was published only included a small number of taxa (Roberts *et al.*, 2011). The combined *L152* dataset from the UoW and TFREC culture has resulted in a sequence dataset of 237 isolates within the *A. alternata* species group. Importantly, this allows comparison of strains used by R.G. Roberts

and E.G. Simmons with those studies using *endoPG* (Peever *et al.*, 2004, Andrew *et al.*, 2009) or *Alta1* (Hong *et al.*, 2005, Lawrence *et al.*, 2011, Lawrence *et al.*, 2013). This may be important if researchers want to use *endoPG* or *Alta1* loci for phylogenetics but want to select a genetically diverse set of representative isolates from the well characterised collection of R.G. Roberts, which contains many E.G. Simmons representative isolates (from Simmons (2007).

The three FUNYBASE loci used, *MS432*, *MS550* and *MS578* showed high levels of variation (5%, 9% and 5% of sites were polymorphic, respectively). The gene *MS432* is a protein of unknown function, *MS550* is involved in cardiolipin synthesis and *MS578* is a subunit of DNA polymerase zeta (Marthey *et al.*, 2008). The fact that these genes are conserved through all fungi suggests they have housekeeping functions, but it is unknown whether they also have a role in pathogenicity. FUNYBASE is shown to be a useful tool to identify novel phylogenetic loci and this highlights the utility of genome sequence data for identifing novel phylogenetic loci, particularly from related genomes to the system of interest, as FUNYBASE loci were generated from conserved sites between *A. brassicicola* and other Dothideomycete species. Design of highly variable primers specifically to the system of interest will allow phylogenetics to be performed with greater resolution in the future. This will be aided by the generation of whole genome sequence data for 12 *A. alternata* isolates, performed in this study. Additionally, due to my primers being designed to conserved sites between Dothideomycete genomes they should be appropriate for phylogenetics in other Dothideomycete systems. This means that these three loci, and primer sets for another 14 loci (Table 3.3), could be used to perform phylogenetics in genera that still have unresolved taxonomies. This would be particularly applicable to closely related genera such as *Embellisia*.

Commonly Used loci

RPB2 was the most variable commonly used phylogenetic locus that was assessed. This locus has been used widely as part of the Assembling the Fungal Tree of Life (AFTOL) project (Lutzoni *et al.*, 2004, Celio *et al.*, 2006) along with *TEF* and rDNA loci. As such, these loci have been used to establish evolutionary relationships within

the Dothideomycete family (Schoch *et al.*, 2006, Schoch *et al.*, 2009b). This includes being used resolve species in other Pleosporales genera such as *Leptosphaerea*, *Melanomma* and in the *Lophiosomataceae* (Schoch *et al.*, 2009b). The *TEF* locus showed low variability within the *A. alternata* species group along with *gpd* and Actin, with less than five SNPs in alignments. These loci have shown species level variability in other systems such as *Fusarium*, *Cochliobolus*, *Stemphylum* and *Verticillium* (Berbee *et al.*, 1999, Camara *et al.*, 2002, O'Donnell *et al.*, 2010, Inderbitzin *et al.*, 2011), but evolutionary relationships between *A. alternata* species group taxa may be closer than those observed in other genera.

Ribosomal DNA loci

Low variability of rDNA loci within the *A. alternata* species group was expected. Sequencing of ITS and LSU rDNA loci has previously been used to identify major sporulation groups in the *Alternaria* genus, but has not shown resolution within the *A. alternata* species group (Pryor and Gilbertson, 2000). Kusaba and Tsuge (1995b) found that all *A. alternata* pathotypes possess identical ITS haplotypes. The ITS region showed limited resolution between *Alternaria* in the pistachio pathosystem where *A. infectoria* could be resolved from *A. alternata*, but no variability was present in the *A. alternata* species group (Pryor and Michailides, 2002). Peever *et al.* (2004) reported that although SSU showed resolution between large and small spored *Alternaria*, it could not resolve small spored species within *A. alternata* species group that caused disease on citrus.

Restriction fragment length polymorphism (RFLP) work has previously found variation in rDNA regions between *A. alternata* isolates from Asian pear in Japan (Adachi *et al.*, 1993, Adachi and Tsuge, 1994). Restriction sites were located within the nuclear rDNA locus and showed variation within and between populations (Adachi *et al.*, 1993, Adachi and Tsuge, 1994). Variability in the rDNA loci used in this study did not support the high levels of variability reported then (Table 3.1). Unfortunately, due to the reported studies not using reference isolates, their results cannot be interpreted in comparison to this work. Their work does indicate that non-tested regions of rDNA may offer higher levels of variation than observed in rDNA

loci in this study. This may include the intergenic spacer (IGS) region, which has been shown to show population level variability in other plant pathogens, such as *Sclerotinia sclerotiorum* (Clarkson *et al.*, 2013). If variability is present in other rDNA regions, they will not necessarily show comparable levels of resolution to highly variable loci identified in this study as rDNA loci are often more conserved than gene coding loci. This trait has been considered beneficial for application as universal fungal barcoding loci (Schoch *et al.*, 2012). However variability is a trait that is required for resolution between closely related taxa. Gene coding loci may offer resolution at lower taxonomic levels and may be used in conjunction with universal barcoding genes. Fungal barcoding databases such as the AFTOL database, and the quarantine pest identification database QBOL use multiple loci to barcode species (Celio *et al.*, 2006, Bonants *et al.*, 2010). Highly variable coding genes were shown to be more appropriate for phylogenetics than rDNA loci. It is recommended that the ITS locus is used for general fungal barcoding and if a sequence is identified as *A. alternata*, one of this study's highly variable regions can be used to identify which major phylogenetic clade (and therefore subspecies) the isolate belongs to.

CHAPTER 4

CHARACTERISING MORPHOLOGY OF *ALTERNARIA*

4.1 INTRODUCTION

The morphological framework of the *Alternaria* genus, as set out by Simmons (2007), recognises 276 *Alternaria* taxa. Recent phylogenetic revisions of the *Alternaria* genus support many of the morphological species described in Simmons (2007) as discrete phylogenetic groups (Lawrence *et al.*, 2013, Woudenberg *et al.*, 2013). However phylogenetic resolution of morphological species has shown limits, as genetic variation within the *A. alternata* species group is reported to not support morphological species concepts (Andrew *et al.*, 2009). There is dispute in the literature as to whether morphological variation within the *A. alternata* species group represents discrete species (Simmons, 1999b, Roberts, 2001, Simmons, 2007), or whether morphological variation is continuous and does not represent discrete species (Nishimura, 1980, Nishimura and Kohmoto, 1983, Kusaba and Tsuge, 1994, Kusaba and Tsuge, 1995b).

Sporulation groups in *Alternaria alternata*

Within the *A. alternata* species group, morphological species may belong to one of six sporulation groups. These sporulation groups were proposed by Simmons and Roberts (1993) following isolations of several hundred *Alternaria* isolates from leaf, fruit and twig lesions of Japanese pear. Upon investigating the patterns of sporulation in these isolates, they were categorised into six sporulation groups. Within these groups, two were considered to represent pathogens with host-specific pathogenicity to pear. These represented Sporulation Groups 1 and 2 (Fig. 4.1), of which Group 2 was considered to represent the pear specific pathogen *A. gaisen*. Sporulation Group 1 isolates were noted to be similar to Group 2 isolates, with some showing evidence of host pathogenicity. However, subsequent literature has not referenced sporulation Group 1. The remaining four Sporulation Groups represented other morphological taxa and this system has formed the basis for a hierarchical classification of the many morphologically described small-spored *Alternaria* spp. (Fig. 4.2).

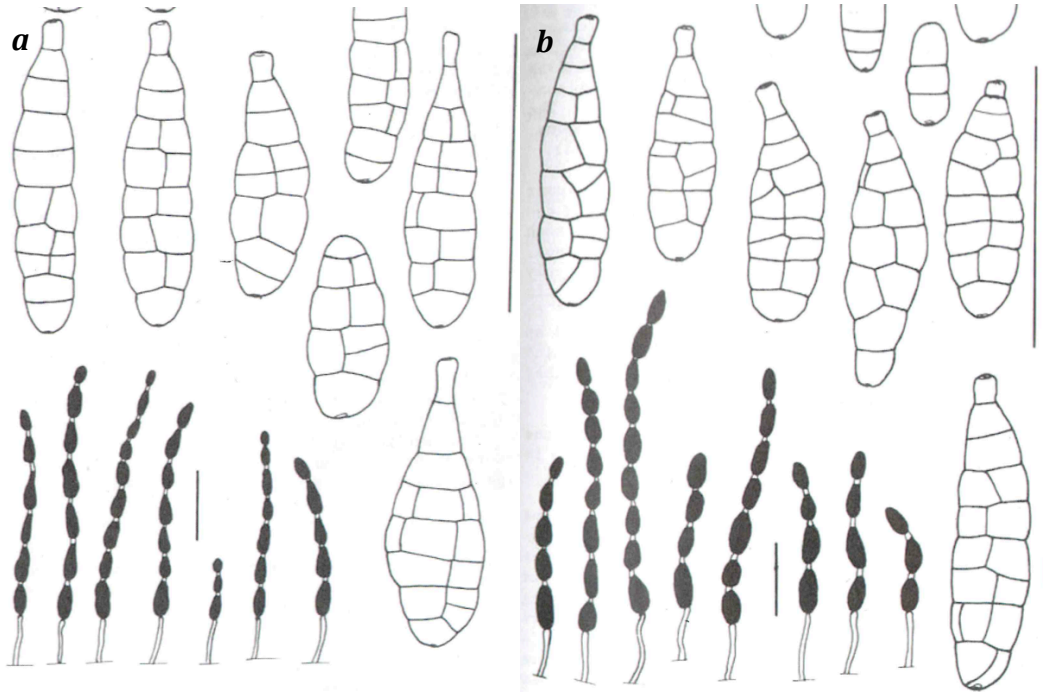


Figure 4.1 Similarity of sporulation characters used to recognise *Alternaria* spp. sporulation groups 1 and 2 as proposed in Simmons and Roberts (1993): Images adapted from Simmons and Roberts (1993) where morphological group 1 (*a*) and morphological group 2 (*b*) were presented as morphologically distinct groups. Clear differences between the two groups were not established in the paper. Scale bar represents 50µm.

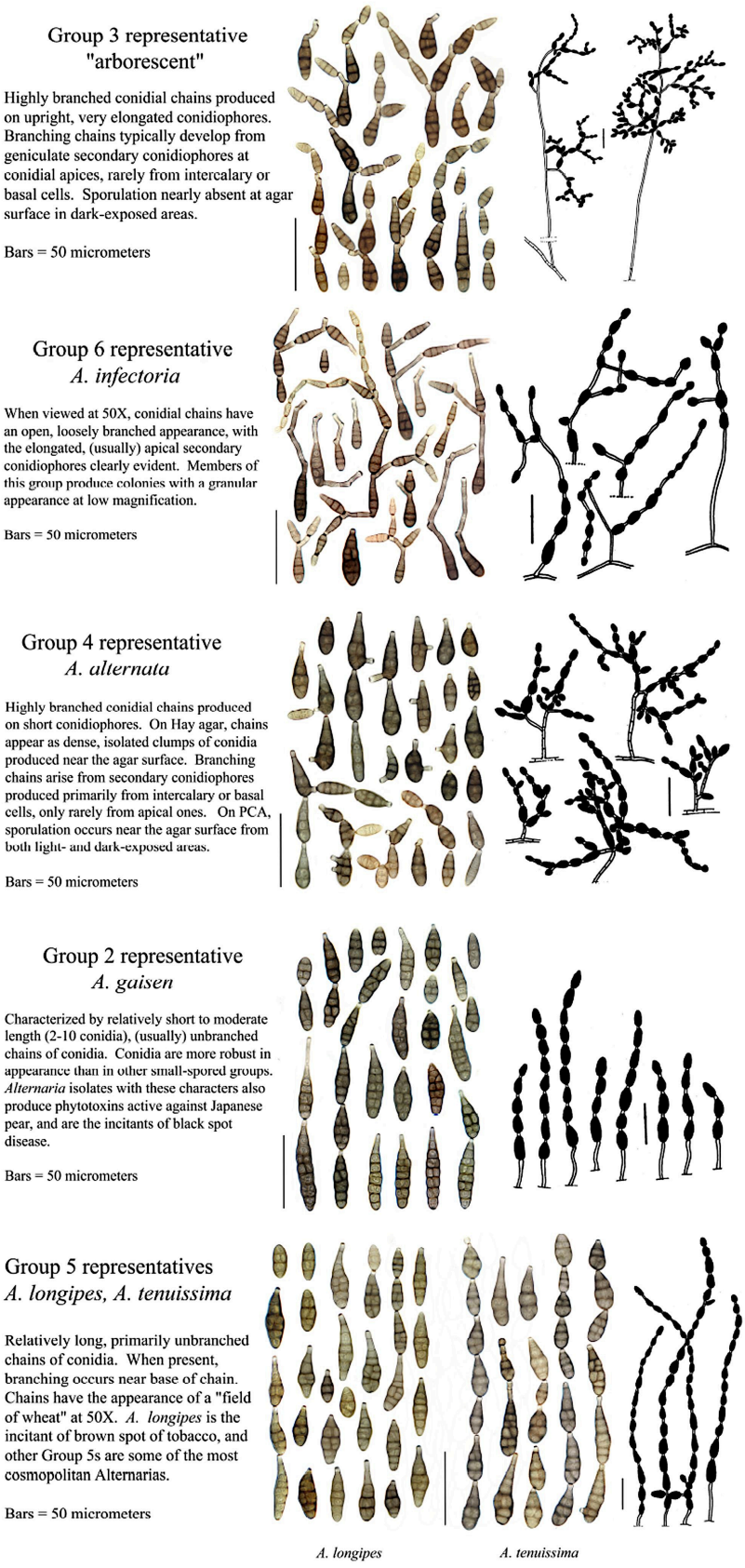


Figure 4.2 Sporulation characters of *Alternaria* spp. sporulation groups 2-6 as proposed in Simmons and Roberts (1993): Scale bar represents 50µm. Image made by R. Roberts (Unpublished) and reproduced with permission.

Specific morphological descriptions

Species descriptions for 246 *Alternaria* morphological species are presented in Simmons (2007). The rationale behind assigning species status to these taxa is stated: '*If the taxon under observation has stable colony development in anexic culture, distinctive sporulation features, and microscopic characters distinguishable from those of similar taxa, then the taxon requires a unique tag to hold its place for retrieval in the published literature*'. Morphologically described, small-spored *Alternaria* spp. in Simmons (2007) are each associated with morphological species groups as are described in Simmons and Roberts (1993). This can be considered as a form of hierarchical classification. It is important to note that this grouping has been specifically stated as describing relationships in morphological characters and not reflecting the evolutionary history of taxa (Simmons, 2007).

Morphological descriptions of *Alternaria* spp. are performed under standard conditions (Simmons, 2007). These consist of seven days growth under short-day light cycle (8 hrs light / 16 hrs dark) at 22 °C, under a gradually drying atmosphere (no humidity control and unsealed plates). Three agars are used for standard identification, potato carrot agar (PCAgar), V8 juice agar or hay agar. PCAgar is the most commonly used agar and most morphologically described species have descriptions of growth on this medium. Brief morphological descriptions, paraphrased from Simmons (2007) are presented below, along with representative drawings (Fig. 4.3). The morphological species that are presented are those that E.G. Simmons thought likely to be represented in the University of Warwick Culture Collection (personal communication):

Alternaria alternata

A. alternata colonies are approximately 40 mm in diameter at seven days consisting of chains of 4-6 conidia on short conidiophores. The typical sporulation pattern comprises a single sub-erect conidiophore and an apical cluster of branching chains of small conidia separated by short secondary conidiophores. Single chains of conidia may have up to 15-20 conidia. Spores at the base of the chain are more ellipsoid with dimensions 25-30 µm x 5-9 µm with transverse septae and a few, or no longisepta.

Subsequent spores are 7-25 µm x 5-12 µm with 1-7 (commonly three) transeptae with few longisepta.

Alternaria arborescens

A. arborescens colonies are approximately 50 mm at seven days consisting of long primary conidiophores bearing a few terminal and sub-terminal branches. Each branch bears a branching chain of conidia. Major conidial chains are 2-6 spores long and typically produce branches of 1-5 conidia. Conidia are short ovoid or ellipsoid. The body of conidia produced on PCAgar is 12-30µm x 7-11 µm with 1-4 transepta and no longisepta or 1-2 longitudinal or oblique septa in a small percentage of the spore population. Secondary conidiophores are small and may be oblescent.

Colonies have dense surface sporulation, with dense multiple branched chains of conidia. Chains are 4-6 conidia long on short conidiophores and 15-20 conidia long in older cultures. Mature conidia are ellipsoid and become more spherical further up the chain, where spores are younger. Typical dimensions of adult spores on PCAgar are 10-30 µm x 5-12 µm. Size ranges of mature conidia are of limited use in differentiating taxa in the species group. Branching occurs from secondary conidiophores arise from the conidium body of cells.

Alternaria gaisen

A. gaisen colonies are approximately 60-70 mm at seven days and consist of chains of 3-9 spores that are usually unbranched but may also produce 1-2 short side branches. Conidia are between 32-45 µm x 14-18 µm and typically have 5-8 transverse septae and 0-1 longitudinal septae in each transverse segment. Each conidium has an apical secondary conidiophore of 1 cell. They do not have a strongly tapered body or a true beak.

Alternaria tenuissima

A. tenuissima colonies are approximately 50 mm at seven days consisting of uncrowded chains of up to 12 conidia on branching hyphae. Fully developed chains produced under standard conditions have patterns of sporulation and conidium morphology of considerable diversity. Typical spores are 32-45 µm x 14-18 µm.

Lower condia in chains have fewer longitudinal septae. Higher conidia have sub-constricting transepta. Hand drawn images show presence of longitudinal septation in higher conidia in chains. Branching within the chain is uncommon.

Alternaria mali

A. mali colonies are approximately 60 mm at seven days consist of chains of 10-15 spores. Branching within the chain occurs but is not common. Branches, when present, produce only a few conidia. Conidia are 30-45 µm x 5-8 µm, usually have 5-8 transverse septa and usually no longisepta or at most 1 longiseptum in 1 or 2 transverse segments of the broadest conidia of a chain. Apical secondary conidiophores may generate a conidiogenous branch.

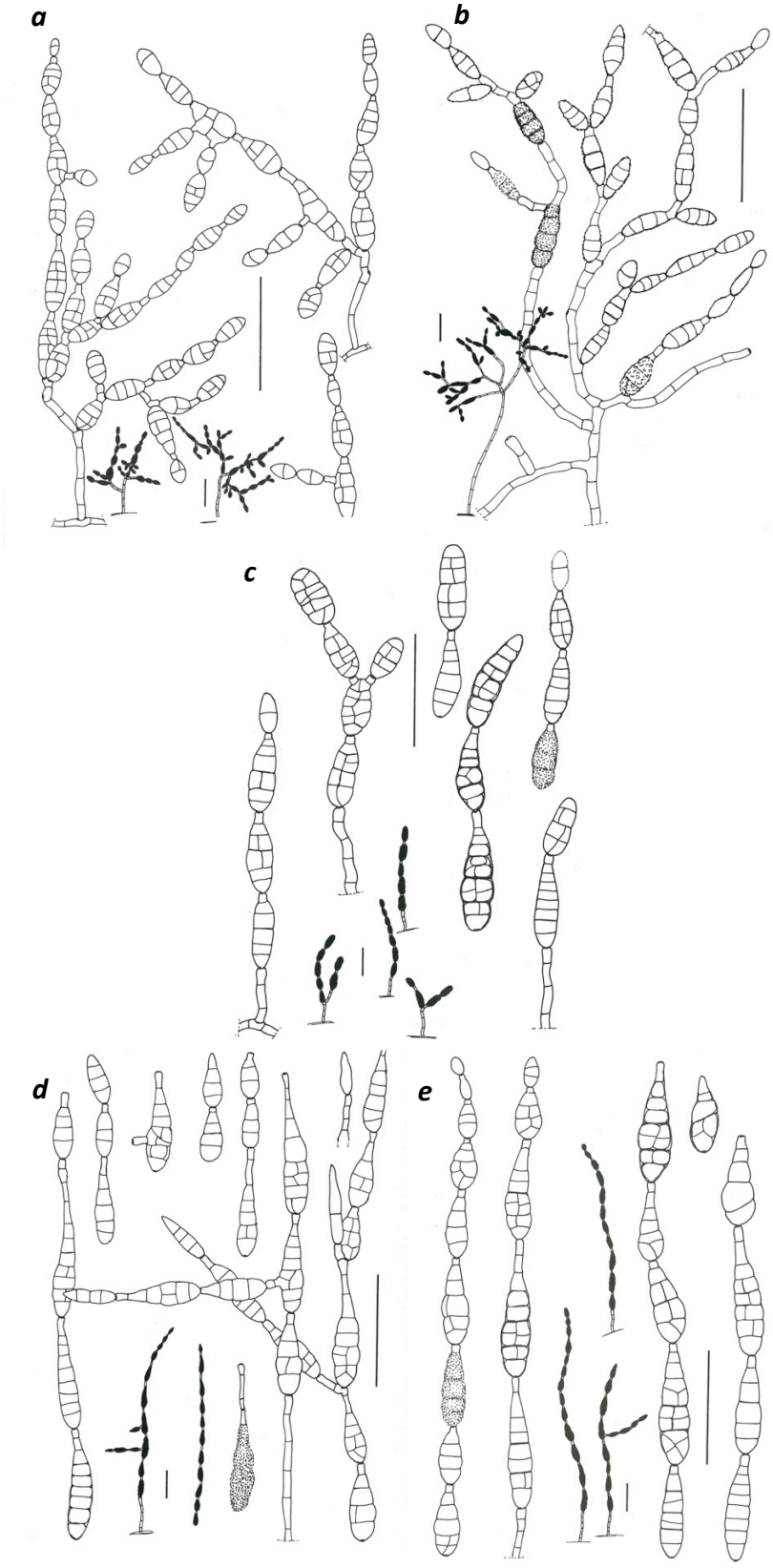


Figure 4.3 Representative morphological drawings of Alternaria species: a) *A. alternata* b) *A. arborescens* c) *A. gaisen* d) *A. mali* e) *A. tenuissima* sporulation patterns and spores as displayed in Simmons (2007). 50µm scale bars are shown. Images reproduced with permission.

Critique of morphological species descriptions

Morphological species descriptions of many small-spored *Alternaria* have been challenged. Morphological characters have been described as being too variable within taxa to be of practical use in identification (Smith *et al.*, 1992). Certainly a lack of clear resolution between taxa has led to recommendations that many of the described small-spored *Alternaria* spp. be considered as a single species *A. alternata* (Andrew *et al.*, 2009).

Morphological species descriptions that are based on a single isolate exhibiting an unusual morphology may lead to there being an overabundance of species. An example of this is shown in the morphological descriptions of *Alternaria* citrus pathogens that occurred during the course of a phylogeography study. As part of this study a group of isolates were submitted to the personal collection of E.G. Simmons to confirm that morphological species identification had been performed correctly. From these isolates, ten new morphological species of *Alternaria* were described (Simmons, 1999a). When phylogenetic analysis was performed on these morphological species the resultant phylogeny, based upon *endoPG* and two other loci, found multiple morphological species were assigned to each phylogenetic clade (Peever *et al.*, 2004). This led to the conclusion that the number of morphological species identified was more than could be supported under a phylogenetic species concept (Peever *et al.*, 2004).

Supporting phylogeny with morphology

Phylogenetic investigations into fungal taxa are showing that many of those previously thought to represent a single morphological species may represent complexes of distinct species (Cai *et al.*, 2011). This has been shown in *Fusarium* and *Colletotrichum*, both plant pathogens with newly recognised phylogenetic species complexes (O'Donnell *et al.*, 2010, Cannon *et al.*, 2012). There is concern that as more fungal pathogen species are described on the basis of molecular techniques then fewer ex-type cultures are deposited in herbaria for later reference (Cai *et al.*, 2011). For this reason it is important to not only identify whether morphological characters

support species as identified using molecular techniques but also to identify type isolates that can be deposited in herbaria and used for future study of these groups.

4.2. AIMS

Many morphological species have been described in the *A. alternata* species group and there is still dispute whether there are distinct morphological groups in the *A. alternata* species group at all. A multi-gene phylogeny was determined in Chapter 3 supporting three distinct clades in *A. alternata* (Fig. 3.2). This chapter aimed to investigate morphological variation in the *A. alternata* species group and to establish whether morphological variation supported the identification of these discrete phylogenetic clades.

Specific aims were:

1. To determine morphological characters of isolates in the *A. alternata* collection and to establish whether different groups can be identified.
2. Identify associations between the morphological characters of isolates and their phylogenetic clade as identified in the multi-locus phylogeny in Chapter 3.

4.3 MATERIALS AND METHODS

Morphological analysis was performed on 112 *A. alternata* isolates (Table 2.1) to investigate whether trends in morphological characteristics observed within *Alternaria* taxa were consistent with the morphological-species descriptions as set out in (Simmons 2007). The experiment was replicated three times.

Cultures were grown on potato carrot agar (PCAgar). The protocol for making PCAgar is described in Simmons (2007): 20 g of white potato and 20 g of carrot were diced and autoclaved in a small amount of distilled water. This slurry was then forced through a fine sieve and water was added to make the solution to 1 litre. 20 g of agar powder (Agar-agar technical, Merck) was added and the solution was autoclaved a second time. Agar plates were poured into 90 mm petri dishes after the solution had cooled to 65°C.

Three filter paper disks from long-term storage (See Chapter 2) were put into an eppendorf for each isolate. Each of these eppendorfs was assigned a blind code by a colleague (Dr. C. Howell, University of Warwick) and all other identifying information removed from the eppendorf. These eppendorfs were returned to long-term storage until required.

The experiment was performed three times, with one week between each repeat. For each repeat one disk of filter paper was taken from each anonymised cultures and each of these was placed in the centre of a PCAgar plate. This was performed to "condition" cultures coming out of long-term storage to experimental conditions. These were grown for seven days in a growth chamber (MLR-350/T, Sanyo) with conditions set to a short-day light cycle (8 hrs light/16 hrs dark) at 22 °C, under a gradually drying atmosphere (no humidity control and unsealed plates). These cultures were used to inoculate a second, "experimental", PCAgar plate. Inoculations were performed using a 5 mm cork borer to remove an agar plug from a region, preferably sporulating, approximately 5 mm within the mycelial margin; this plug was placed, facing upwards, in the centre of the "experimental" PCAgar plate. Experimental plates were grown using the same culturing conditions as described above.

Morphological data was recorded seven days after inoculation. Colony diameter was measured and sporulation characteristics further investigated through microscopy. To investigate spore morphology for each of the isolates at slides were made, as described by in Simmons (2007): In summary, transparent adhesive tape was used to collect spores from the colony surface, this was put on a glass microscope slide and a few drops of lactic acid were added before a cover slip was placed over the slide and the slide gently heated to fix spores.

Images were taken at three locations for each slide at 500 times magnification using a camera (C5050 Zoom, Olympus) attached to a microscope (SZX7, Olympus). The three locations were at approximate positions of 25%, 50% and 75% across the length of the slide. These sporulation images were later analysed using Image J (Abramoff *et al.*, 2004). Before performing image analysis on spore photos, a calibration photo was loaded with a known distance between two points. This was used to set all measurements made in that session to a known number of pixels.μm^{-1}. The calibration photo was of gridlines on a haemocytometer (Improved Neubauer, Webb England), with a known distance of 200 µm between points. The following morphological characters were recorded for each microscope image: 1) The number of conidia present in the image; 2) The number of a) conidiophore b) intercalary c) basal branches present in the image (Fig. 4.4.*a*); 3) The number of conidia making the longest conidial chain of conidia were counted (Fig. 4.4.*b*). 4) A representative selection of ten spores were then chosen from the image and the following characters measured: a) conidial length b) conidial width c) number of transverse septae d) number of latitudinal septae (Fig. 4.4.*c*).

Figure 4.4 Characters used in morphological analysis. *a*) branching patterns: Spores demonstrating *a*) apical branching, *b*) intercalary branching and *c*) basal branching. ***b*) longest conidial chain:** The morphological character *longest conidial chain* was defined as the number of spores between the base and the ap*ex*. In this case it would be three. ***c*) spore characters:** Spore characters of *a*) length *b*) width *c*) no. transverse septae *d*) no. longitudinal septae marked on an example spore by coloured arrows. Spore images adapted with permission from Roberts (unpublished).

Mean values were calculated for each morphological character. Different morphological characters had different number of replicates / pseudo-replicates from which these means were taken: Colony size had a mean calculated from three replicates; Means taken from slide images such as number of conidia were taken from nine measurements (three pseudo-replicates within three replicates); means for individual spore measurements (such as spore size) were calculated from 90 measurements (10 spores in each of three pseudo-replicates, within three repeats).

When an isolate did not show presence of any spores on slide images in two replicates then it was removed from the dataset. Following processing of the dataset the blind-codes for each of the isolates were removed and the isolates renamed.

A principal components analysis was performed to explain variation within the dataset using Genstat 13 (GenStat, 2011). Data from the ten morphological variables was used to form a correlation matrix. Ten principal components were calculated and a scree plot was made to show the relative contribution of each of these to summarising the data. From this the most informative components were selected and investigated for biological meaning.

Association of isolates with similar morphological characters was investigated through constructing a dendrogram using Genstat 13 (GenStat, 2011). A Euclidean correlation matrix was calculated and the dendrogram determined using the furthest neighbour algorithm. Clades that were present at a 95% similarity level were considered representative of major groupings within the dendrogram and were used for further analysis. The Euclidean matrix was calculated from those principal components that were considered to reflect relevant biological meaning. A constant data range was used to form the Euclidean matrix, this was equal to the largest range between a maximum and minimum principal component score observed within one of the principal components within the dataset. This allowed the relative weightings of principal components to be maintained within the matrix.

Principal component scores were plotted against each other across the dataset. Individual data-points were identified by the morphological clade they were associated with. Clades were identified that were positively or negatively associated with each principal component. This allowed morphological characters to be assigned to each clade on the basis of the weightings of each morphological character to each

principal component. The resultant morphological characters could then be related to morphological species descriptions.

Concordance between clades identified within the morphological dendrogram and phylogenetic clades, as in a multi-locus phylogeny (Fig. 3.2), was examined (this was done through labelling phylogenetic clades on the morphological dendrogram).

4.4 RESULTS

Data was collected to explore whether trends in morphological characteristics were consistent with morphological species descriptions as set out in Simmons (2007). Many of the 112 isolates did not sporulate in all three repeats of the experiment. The analysis was based upon the 68 isolates that did sporulate in all three repeats.

Principal components analysis (PCA) was used to explore the variation and association in morphological characters. Ten principal components were calculated for the dataset. Successive principal components explained progressively less of the variation within the dataset, with a marked decline after the sixth principal component (Fig. 4.5). Of the ten components, the first five summarised over 85% of variation in the dataset (Table 4.1).

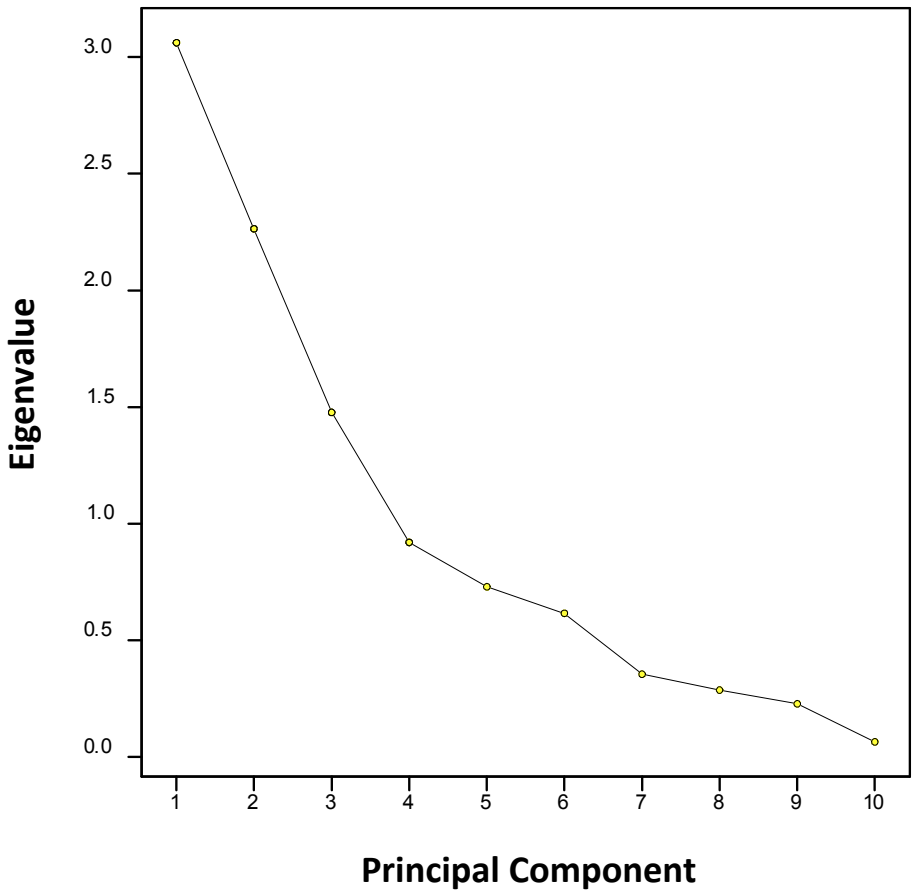


Figure 4.5 Principal components analysis scree plot: Scree plot of Eigenvalues for each of the ten principal components (Root) calculated from morphological characters. The extent to which principal components explain the data drops after the sixth principal component.

Exploring the first principal component

All coefficients in the first principal component contributed positively to the score for an individual (Table 4.1). This meant that morphological characters were not being contrasted against each other but that variance in the size of the data was being explained, contrasting individuals with high values for morphological characters against individuals with low values for morphological characters. Strong weightings were put on individuals that had large values for numbers of condia, maximum chain length and presence of branching. These are characters that would be expected to be high if an isolate sporulated well. This was considered to imply that isolates were separated on the basis of how well they sporulated.

Table 4.1 Contribution of morphological characters to principal components: Variation and loadings accounted for by each of the first six principal components (PC1-6). The relative contribution of each morphological character to a principal component is also presented. The percentage of the variation total variation in the dataset that each component explains is shown.

Morphological character	PC1	PC2	PC3	PC4	PC5	PC6
Variation (%)	30.61	22.64	14.77	9.2	7.29	6.16
Colony diameter	0.018	0.305	0.208	0.772	0.444	0.206
No. conidia	0.454	0.225	0.038	0.064	-0.271	0.076
Longest chain	0.367	-0.060	0.396	0.081	0.117	-0.608
Conidia length	0.194	-0.552	0.005	0.304	-0.236	0.249
No. transepta	0.126	-0.580	0.166	0.246	-0.181	-0.055
Conidia width	0.269	-0.009	-0.639	0.164	0.034	0.272
No. longisepta	0.236	-0.184	-0.527	0.008	0.455	-0.465
No. basal branches	0.479	0.038	0.105	-0.290	0.045	0.287
No. intercalary branches	0.448	0.072	0.211	-0.269	0.285	0.239
No. conidiophore branches	0.216	0.415	-0.172	0.238	-0.580	-0.294

Determining a morphological dendrogram

The first principal component was considered to not represent the evolutionary history of an isolate, but instead to represent natural variation in sporulation ability. Variation attributed to the first principal component was therefore discarded and the biological meaning associated with the remaining variation was investigated. This was done through calculating a Euclidean matrix from the second to tenth principal component, and determining a dendrogram from this matrix.

A dendrogram was constructed using morphological data from 68 isolates. Nine morphological clades were identified when a 95% similarity cut-off was applied (Fig. 4.6: morphological clades *A*-*E*).

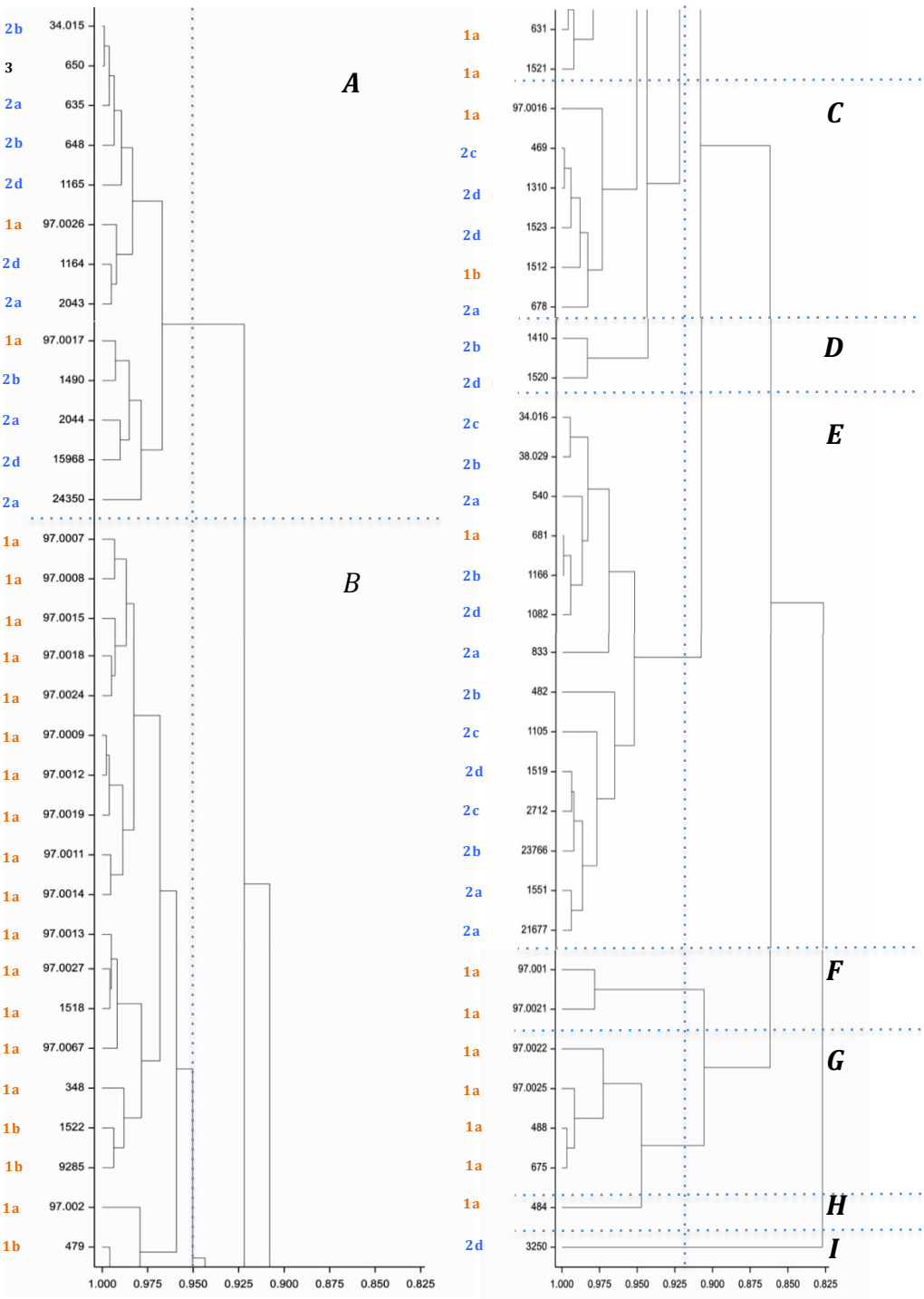


Figure 4.6 Dendrogram determined from *Alternaria* spp. morphological characters: Morphological dendrogram determined from principal components 2-10. Major clades are identified at a 95% similarity level (*A-I*). Isolates are marked with the phylogenetic clade they belong (*1a-3*) to as identified by the multi-locus phylogeny in Chapter 3 (Fig. 3.2).

Values for morphological characters fell within morphological species descriptions. The number of spores on the longest chain was an exception to this, with average values between 2 and 5 conidia long. Species descriptions of morphological species in the *A. alternata* species group would expect chain sizes of approximately 12-15 condia long for *A. tenuissima* and *A. mali*.

Table 4.2 Mean values of morphological characters associated with *Alternaria* spp. morphological clades: Mean values of morphological characters associated with each of the nine clades (*A*-*I*) identified in a dendrogram (Fig. 4.6) determined from the same dataset of morphological characters.

Morphological Characters	Clade *A*	Clade *B*	Clade *C*	Clade *D*	Clade *E*	Clade *F*	Clade *G*	Clade *H*	Clade *I*
Colony diameter	64	67	63	57	67	69	68	65	40
No. conidia	32	47	42	31	50	50	55	92	39
Longest chain	3.4	3.2	2.9	3.7	3.9	3.7	3.0	3.1	3.7
Conidia length	23.9	19.4	20.4	19.3	20.9	19.5	17.5	20.5	22.6
No. transepta	3.4	2.7	2.7	3.0	3.0	2.7	2.1	2.9	3.1
Conidia width	7.4	7.3	7.2	6.4	6.5	8.2	7.5	7.2	7.9
No. longisepta	0.1	0.1	0.1	0.1	0.1	0.4	0.0	0.1	0.3
No. basal branches	0.21	0.31	0.85	0.72	0.42	0.22	0.33	0.33	0.67
No. intercalary branches	0.39	0.48	1.02	0.89	1.08	1.06	0.44	0.00	0.44
No. conidiophore branches	0.30	2.52	1.00	0.33	0.83	2.11	1.88	6.22	0.67

Characterising isolate morphology

Morphological characters associated with each principal component were identified from Table 4.2. Positive or negative association of a morphological clade with a principal component was identified by plotting principal component scores against each other for each of the isolates in the dendrogram (Fig. 4.7-Fig. 4.10). From this, the morphological characters associated with each phylogenetic clade were identified.

Principal component 2 (Fig. 4.7)

The second principal component separated isolates with a greater occurrence of branching from conidiophores, shorter, more rounded spores with few transverse septa. This contrasted against isolates with longer spores, with more transverse and longitudinal septae, with a low occurrence of conidiophore branching. Morphological clades *B* and *G* were positively associated with these characters whereas morphological clades *A* and *I* were negatively associated with these characters. These associations are reflected in representative spore images of isolates from these clades (Fig. 4.7). Isolates positively associated with the second principal component may be considered to possess a more *A. arborescens*-like morphology, whereas isolates negatively associated with the second principal component may be considered to possess a more *A. alternata* or *A. tenuissima* -like morphology. We can therefore consider clades *B* and *G* to be more *A. arborescens* -like and clades *A* and *I* to be more *A. tenuissima* and *A. mali* -like.

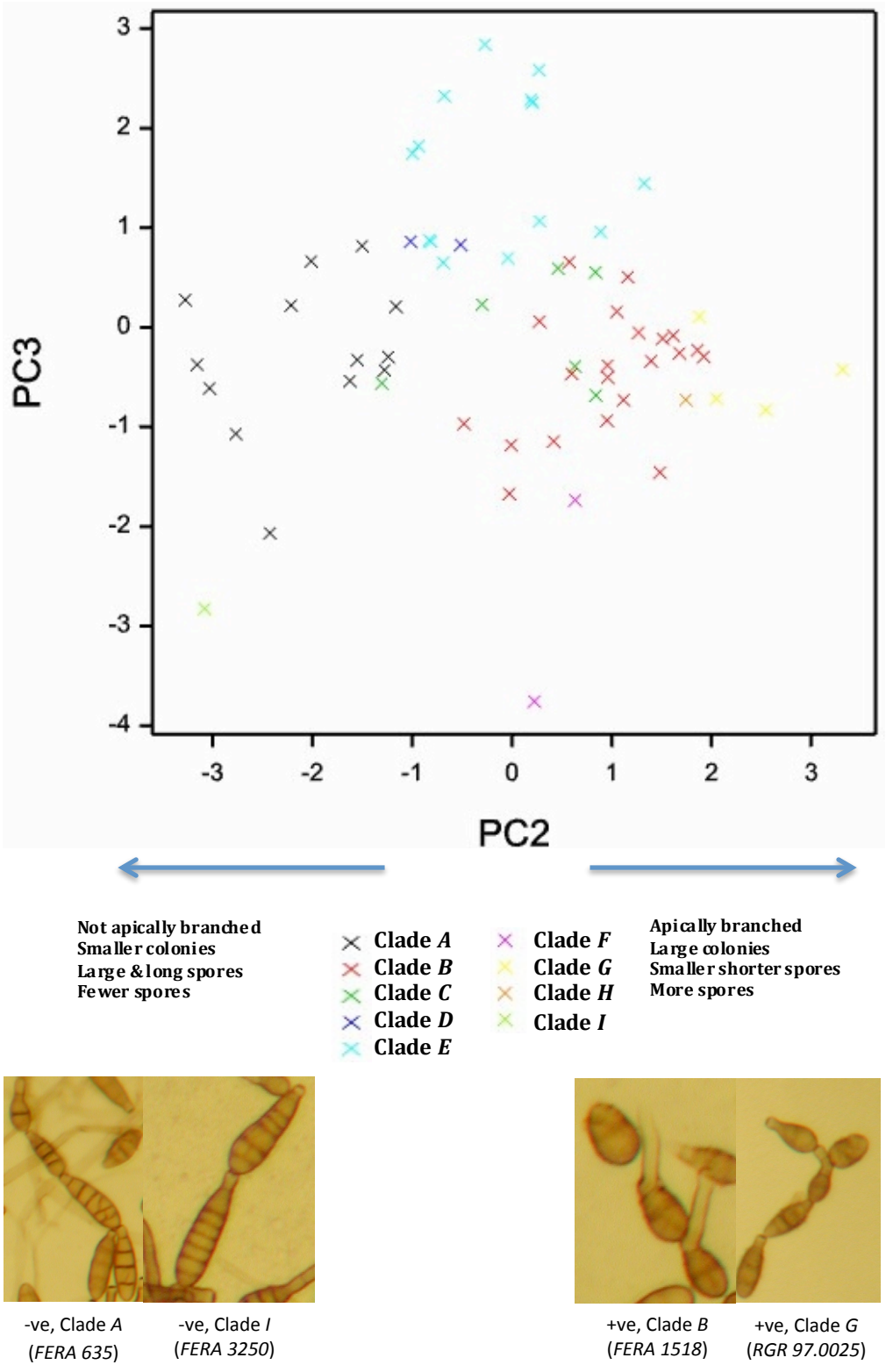


Figure 4.7 Identification of morphological clades associated with principal component 2: Plot of principal component scores for isolates coloured by morphological clade (as determined in Fig. 4.6). Clades positively or negatively associated with principle component 2 (PC2) are associated with the morphological characters listed beneath the plot. Principal component 3 (PC3) is plotted to aid identification of clades. Representative images are presented for clades positively or negatively associated with PC2.

Principal component 3 (Fig. 4.8)

Isolates that were positively associated with the third principal component had longer chains of spores. Their conidia had more transeptae, were thinner, with a lower occurrence of longisepta, were rarely branched from conidiophores but had more branching from within condia. These characters may be considered to be representative of *A. tenuissima* and *A. mali* -like morphologies. Isolates negatively associated with the third principal component had shorter chains with fewer transeptae, were wider with more longitudinal septation and had greater degrees of conidiophore branching but with less branching from within conidia. These characters represent a more *A. arborescens*-like morphology. Morphological clade *E* was positively associated with the third principal component and morphological clade *F* was negatively associated with the third principal component. Representative images of spores from isolates within these clades reflect these patterns (Fig. 4.8). Morphological clade *E* may be considered more *A. tenuissima* and *A. mali* -like whereas morphological clade *F* may be considered more *A. arborescens*-like.

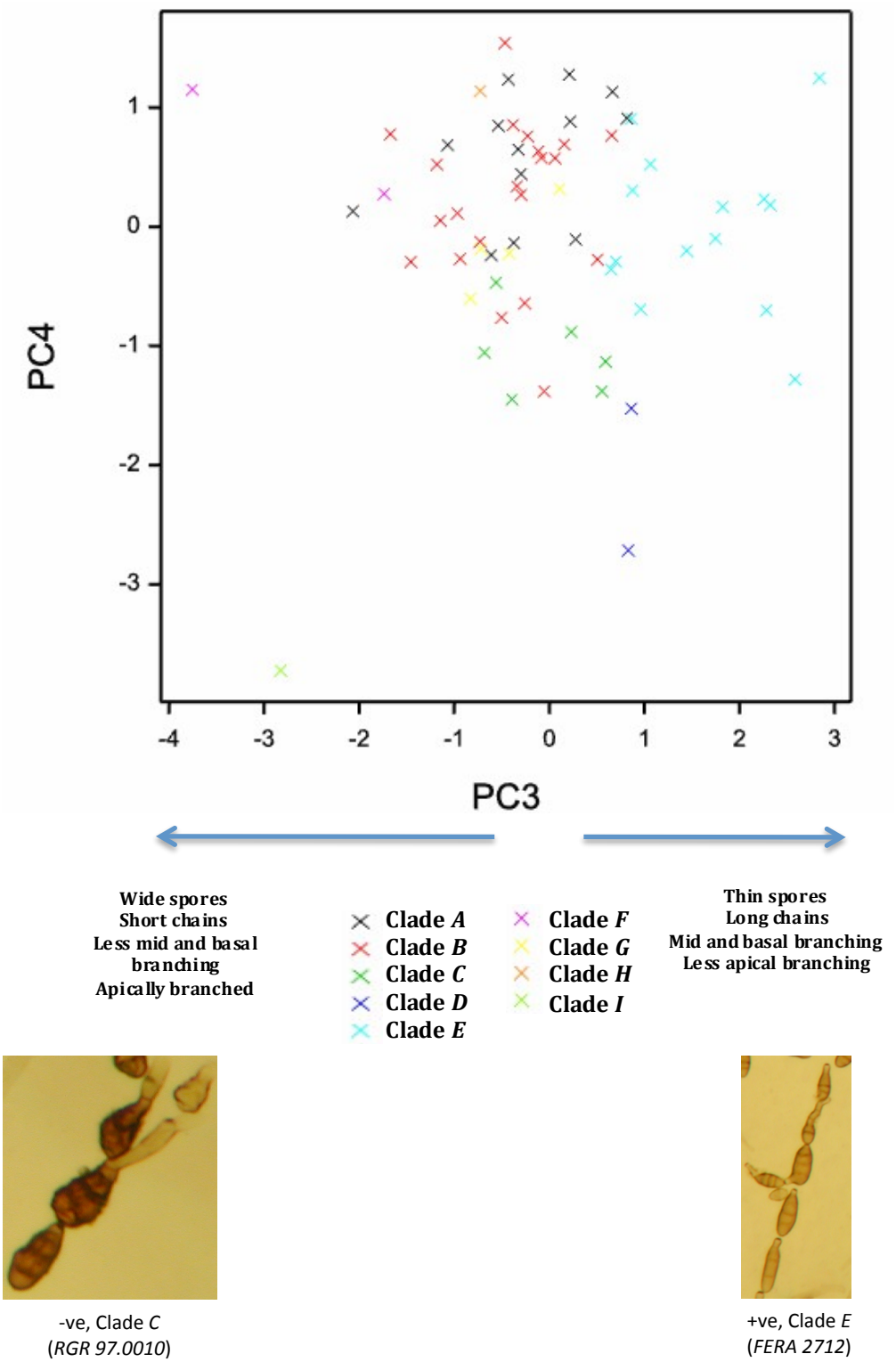


Figure 4.8 Identification of morphological clades associated with principal component 3: Plot of principal component scores for isolates coloured by morphological clade (as determined in Fig. 4.6). Clades positively or negatively associated with principle component 3 (PC3) are associated with the morphological characters listed beneath the plot. Principal component 4 (PC4) is plotted to aid identification of clades. Representative images are presented for clades positively or negatively associated with PC3.

Principal component 4 (Fig. 4.9)

The fourth principal component put a strong positive weight on colony size and contrasted this, and apical branched spores, against basally and mid-branching spores. Morphological clades *C* and *D* were negatively associated with this principal component. No morphological clades showed positive association with this principal component. Clades *C* and *D* had smaller colonies, smaller spores, branching within spores and less conidiophore branching. Representative spores of isolates within these clades reflect these patterns (Fig. 4.9). Patterns of morphological association were considered to reflect an *A. alternata* -like morphology.

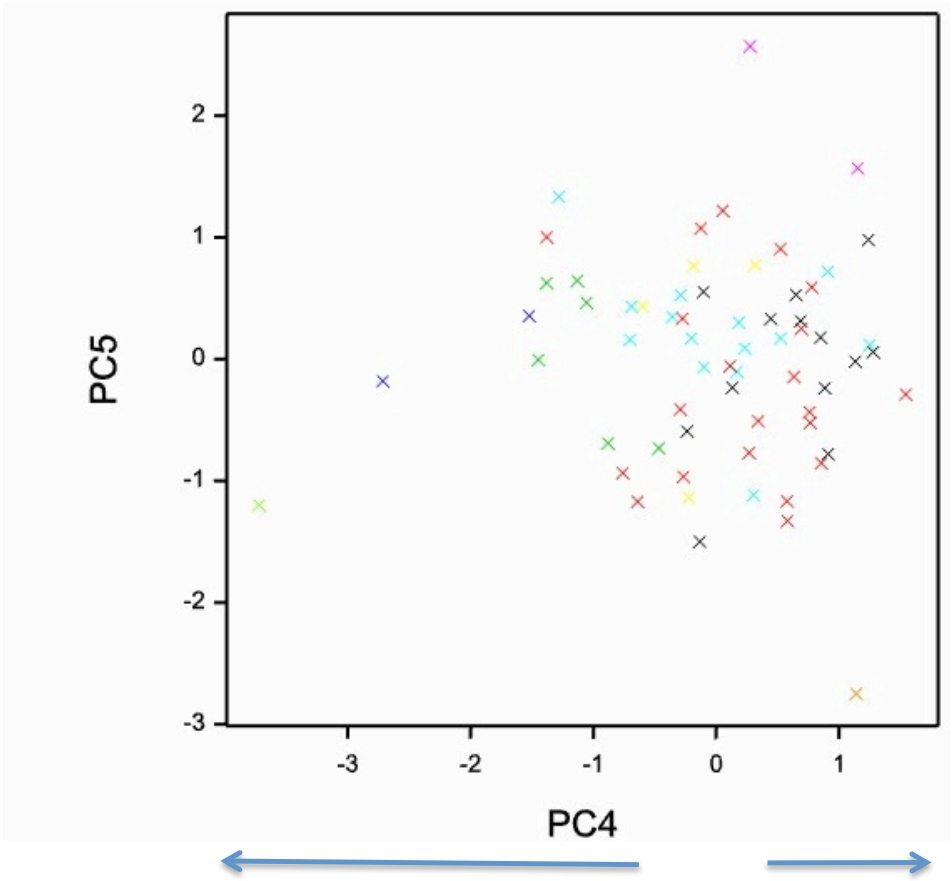


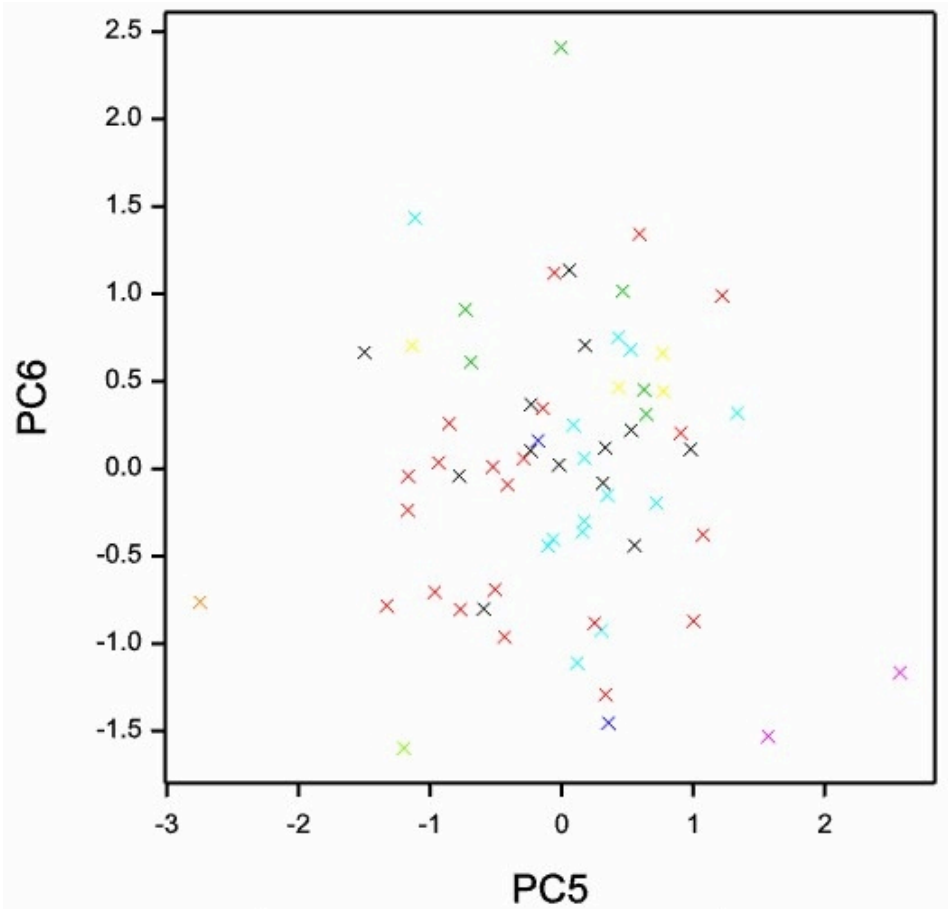
Figure 4.9 Identification of morphological clades associated with principal component 4: Plot of principal component scores for isolates coloured by morphological clade (as determined in Fig. 4.6). Clades positively or negatively associated with principle component 4 (PC4) are associated with the morphological characters listed beneath the plot. Principal component 5 (PC5) is plotted to aid identification of clades. Representative images are presented for clades negatively associated with PC4.

Principal component 5 (Fig. 4.10)

Isolates that were positively correlated with the fifth principal component had larger colonies, fewer spores, smaller spores, more longisepta and more intercalary branching. Isolates that were negatively correlated with the fifth principal component had smaller colonies, more conidia, larger spores with fewer longisepta had less branching from within spores but had more conidiophore branching. Morphological clade *F* was positively correlated with the fifth principal component and morphological clade *H* was negatively associated with the fifth principal component. Images of representative spores from isolates within these clades reflected these patterns (Fig. 4.10). These morphological characters were not considered to represent any morphological species descriptions.

Principal component 6 (Fig. 4.10)

The sixth principal component did not resolve morphological clades as identified by the morphological dendrogram. This means that any clades separated by this component were below the 95% similarity cut-off in the morphological dendrogram. This principal component and principal components explaining smaller percentages of variation were discarded.

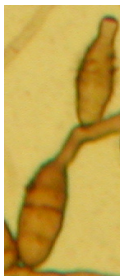


Small Colonies
More spores
Longer spores
Few longisepta
Few intercalary branches
Many conidiophore branches

Clade *A*
Clade *B*
Clade *C*
Clade *D*
Clade *E*
Clade *F*
Clade *G*
Clade *H*
Clade *I*

Large colonies
Fewer spores
Shorter spores
Many longisepta
Intercalary branching
Few conidiophore branches

-ve, Clade *H*
(*FERA 484*)



+ve, Clade *F*
(*RGR 97.0010*)

Figure 4.10 Identification of morphological clades associated with principal component 5 and 6: Plot of principal component scores for isolates coloured by morphological clade (as determined in Fig. 4.6). Clades positively or negatively associated with principle component 5 (PC5) are associated with the morphological characters listed beneath the plot. Representative images are presented for clades positively or negatively associated with PC5. No clades are resolved by principal component 6 (PC6) that have not been resolved by other components.

Comparison with morphological species descriptions

A. arborescens

Morphological clades *B*, *F, G* and *H* displayed *A. arborescens* characters, particularly rounder spores and more conidiophore branching, characters that were consistent with sporulation group 3 as described in Simmons and Roberts (1993; Fig. 4.2) and *A. arborescens* as specifically described in Simmons (2007; Fig. 4.3).

A. tenuissima / *A. mali*

Clades *A*, *E* and *I* displayed morphological characters which could be attributed to sporulation group 5 as described in Simmons and Roberts (1993; Fig. 4.2) and *A. tenuissima* or *A. mali*, as described in Simmons (2007; Fig. 4.3). The representative isolates for *A. mali*, *A. tenuissima* and one of the two *A. alternata* isolates were present in these morphological clades.

A. alternata

Morphological clades *C* and *D* were considered to show morphological characters that could be assigned to sporulation group 4 as described Simmons and Roberts (1993; Fig. 4.2) and A. *alternata*, as described in Simmons (2007; Fig. 4.3). The A. *alternata* reference isolate described by E.G. Simmons (*EGS 34.016*) was present one of these morphological clades.

Association with phylogenetic clade

The association between morphological characters and phylogenetic clade was examined. This was done by plotting the phylogenetic clade that an isolate belonged to (Fig. 3.2) onto the morphological dendrogram (Fig. 4.6). Phylogenetic identification of major clades as distinct groups (Clades 1 and 2) was supported in the morphological dendrogram. Each morphological clade that was identified (*A*-*I*) largely contained isolates of a single phylogenetic clade. No clear morphological

patterns were observed between isolates from minor phylogenetic clades (1*a*-1*b*, 2*a*-2*e*).

Phylogenetic Clade 1 - *A. arborescens*

Morphological clades *B*, *F, G* and *H* contained isolates exclusively from phylogenetic Clade 1 (Fig. 4.11). These morphological clades typically had characters associated with *A. arborescens*. The phylogenetic clade that these isolates belonged to was noted to contain the *A. arborescens* reference isolate (Fig. 4.11). The association of phylogenetic Clade 1 with morphological characters consistent with those of an *A. arborescens* morphology supports the designation of this group as a distinct sub species in the *A. alternata* species group.

Phylogenetic Clade 2 - *A. tenuissima*

Morphological clades *A*, *C*, *D*, *E* and *I* showed association with phylogenetic Clade 2 (Fig. 4.11). Each of these representative isolates was present in phylogenetic Clade 2. Reference isolates for *A. tenuissima*, *A. mali* and *A. alternata* were all present in this phylogenetic clade (Fig. 4.11).

Clades *A*, *E* and *I* were associated with a morphology consistent with *A. tenuissima* and *A. mali* and represented 28 isolates. Clades *C* and *D* were associated with an *A. alternata* morphology and represented eight isolates. This shows that morphologies consistent with *A. tenuissima* and *A. mali* are more prevalent than *A. alternata* in phylogenetic Clade 2. As such it would be inappropriate to name this clade as *A. alternata* as that implies that isolates in this clade have morphologies that are not consistent with these findings. It would also not be inappropriate to name this clade as *A. mali* as this implies that all isolates contained within this phylogenetic clade are apple (*Malus domestica*) pathogens. Due to the high prevalence of *A. tenuissima* morphology and it being inappropriate to name the clade as *A. alternata* or *A. mali*, phylogenetic Clade 2 is named as *A. tenuissima*.

Phylogenetic clade *3* - *A. gaisen*

The isolate representing phylogenetic Clade *3* did not carry a distinct morphology and was grouped within morphological clade *A* (Fig. 4.11). In the phylogenetic analysis

three isolates belonged to this phylogenetic clade. Only one of these three isolates sporulated sufficiently for data to be collected for morphological analysis. A single isolate was considered to be too small a sample size to determine whether the morphology of *A. gaisen* was distinct from other taxa.

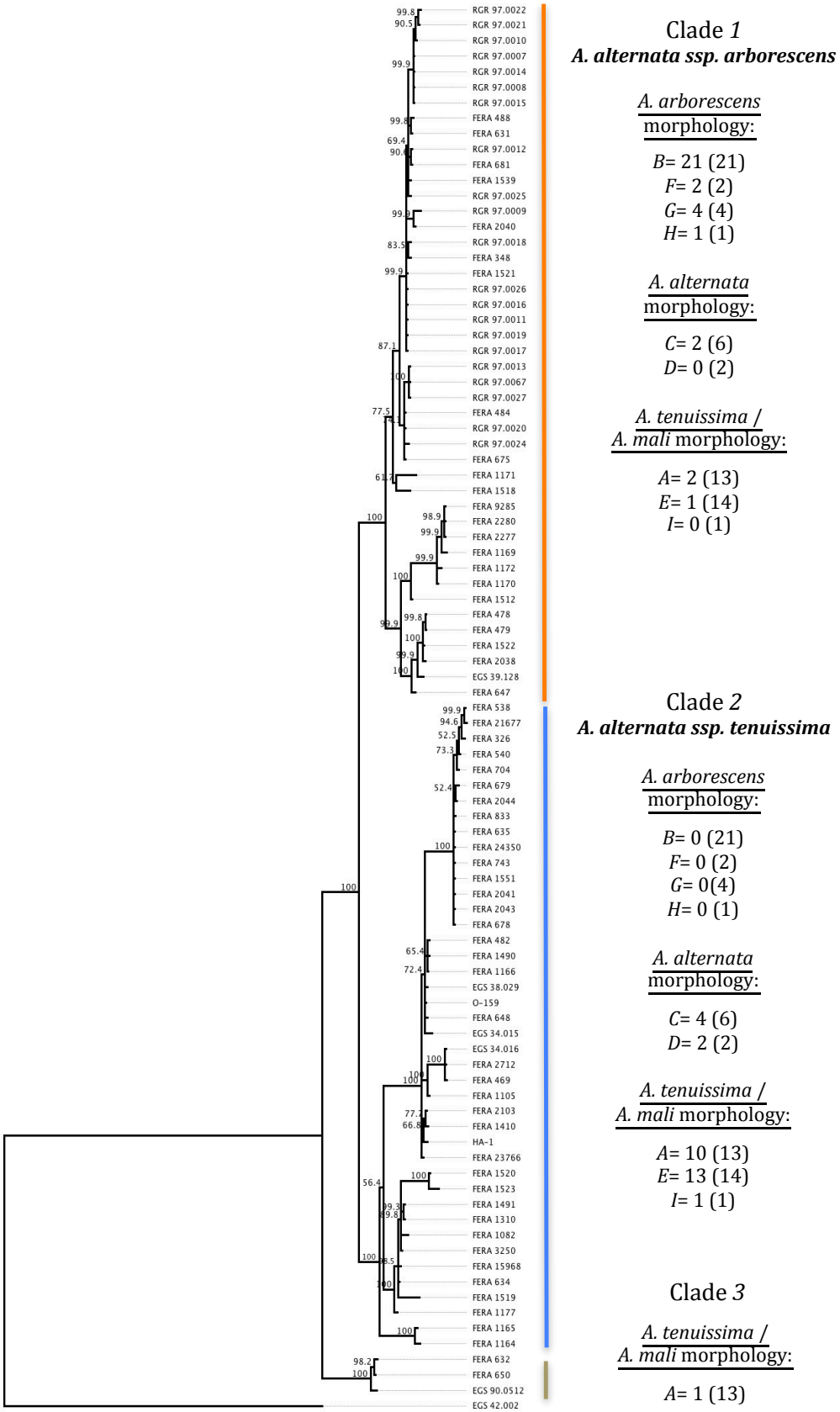


Figure 4.11 Association of morphological clades with phylogenetic clades of the multi-locus phylogeny from Chapter 3 (Fig. 3.2): Major phylogenetic clades (*1-3*) annotated with the number of isolates from each morphological clade (*A-I*) present in that clade (brackets show the total number of isolates present in that morphological clade).

4.5 DISCUSSION

Morphological species recognition

Morphological species can be considered as '*the smallest groups that are consistently and persistently distinct and distinguishable by ordinary means*' (Cronquist, 1978). Morphological data collected from 68 *A. alternata* isolates was used to identify nine morphological clades (Fig. 4.6). Four clades were associated with morphological characters similar to those of the morphologically described species *A. arborescens* (*B*, *F, G* and *H*). Three clades were associated with morphological characters similar to those of the morphologically described species in sporulation group 5 Simmons and Roberts (1993; Fig. 4.2): *A. tenuissima* and *A. mali* (Clades *A*, *E* and *I*). Two clades were associated with the morphological species descriptions for *A. alternata*. This shows that groups of isolates can be identified that show morphological characters similar to those described in the sporulation groups of Simmons and Roberts (1993) and species descriptions of Simmons (2007).

The morphological clades in this study were not identified by what Cronquist (1978) might consider as '*ordinary means*'. A high level of variation was observed between sporulation ability of isolates in the University of Warwick (UoW) culture collection. The effect of this variation was identified through PCA by the first principal component. This component had to be removed from the dataset before a dendrogram could be constructed to investigate the morphological variation associated with evolutionary history. Furthermore multiple clades were associated with each morphological species. As such, this data could not identify *A. arborescens*, *A. alternata* and *A. tenuissima* morphologies as consistent, persistently distinct or distinguishable by ordinary means (Cronquist, 1978). Using these criteria they cannot be considered as morphological species.

Morphological species recognition as performed in the *Alternaria* genus and described in Simmons (2007), states that: '*If the taxon under observation has stable colony development in anexic culture, distinctive sporulation features, and microscopic characters distinguishable from those of similar taxa, then the taxon requires a unique tag to hold its place for retrieval in the published literature'*. If this

framework was applied to this study then up to nine morphological species could have been supported by the dendrogram constructed from morphological data. This highlights a problem with morphological species identification that can be demonstrated using the following example: Two cultures of the same genotype may sporulate differently, and do so consistently. If these two individuals were observed independently using morphological species concepts then they might be considered as separate species. Work by Aoki *et al.* (2009) shows that infection with a dsRNA mycovirus can affect *A. alternata* sporulation ability. Processes such as this leading to inconsistency between isolate sporulation patterns may lead to more morphological species descriptions than can be supported within a phylogenetic framework. This may have occurred in the description of morphological species in the *A. alternata* species group. *Alternaria* sporulation groups 1 and 2 are morphologically similar (Fig. 4.1). The same can also be considered for *A. tenuissima* and *A. mali* (Fig. 4.3), which are both members of sporulation group 5. When performing phylogenetics on morphologically described species causing disease on citrus Peever *et al.* (2004) found that there were more morphological species than could be supported by phylogenetic species concepts.

Isolate morphology supports phylogenetic lineages

Morphology is increasingly being used to support phylogenetic identification of lineages. Revision of the Dothideomycete taxa at the Class level has supported phylogenetic identification of lineages with morphological descriptions (Zhang *et al.*, 2009). This approach has allowed the confident placement of ten previously unplaced species (on the basis of morphology alone) into the fungal tree of life with clades being supported by morphological data (Crous *et al.*, 2009). In the *Alternaria* genus Woudenberg *et al.* (2013) applied morphological species descriptions to each of the *Alternaria* lineages they identified, including "*Alternaria* section *alternaria*" (discussed below). Morphological clades identified in Figure 4.7 gave strong support to the lineages identified by genealogical concordance species recognition (GSR; Fig. 4.11):

Phylogenetic Clade 1 represents *A. alternata* ssp. *arborescens*

Isolates in phylogenetic Clade 1 were identified as carrying spore morphologies consistent with *A. arborescens* (Fig. 4.11). As such this clade was identified as a distinct sub-species *A. alternata* ssp. *arborescens*.

Phylogenetic Clade 2 represents *A. alternata* ssp. *tenuissima*

Morphological data was collected for 30 isolates in phylogenetic Clade 2, of which 24 had an *A. tenuissima* / *A. mali* -like morphology and six had an *A. alternata*-like morphology. Results from this study agree with E.G. Simmons who considered the morphological species *A. alternata* to be rare in the environment (Simmons, 1999b). This opinion was built on similar findings to these, such as those presented in a study of 349 *Alternaria* isolates isolated from pear and apple lesions, where only seven isolates were identified as having *A. alternata* morphologies (despite most of these being identified as being non-pathogens) (Simmons and Roberts, 1993). Due to the low prevalence of *A. alternata* morphologies in the environment and a lack of phylogenetic resolution for this group (Chapter 3) this study considers that the *A. alternata* morphological species does not represent a distinct evolutionary group. In fact it represents a combination of morphological characters possible within the continuous distribution of characters associated with *A. alternata* ssp. *tenuissima*.

Implications of naming lineages as subspecies

Recent revisions of the *Alternaria* genus need to be considered when naming phylogenetic Clades 1 and 2 as subspecies of *A. alternata*. Lawrence *et al.* (2013) performed phylogenetics on 176 species representing *Alternaria* and related genera *Stemphylum*, *Embellisia*, *Nimbya* and *Ulocladium* using five phylogenetic loci identifying eight phylogenetic lineages within the *Alternaria* genus and assigning them the taxonomic rank of section (Lawrence *et al.*, 2013). One of these represented the *A. alternata* species group and was named *Alternaria* section *alternaria*. A similar study by Woudenberg *et al.* (2013) also performed phylogenetics on *Alternaria* and related genera using 123 isolates over three phylogenetic loci. They supported the

designation of *A. alternata* as a section. However it seems that Woudenberg *et al.* (2013) misinterpreted Lawrence *et al.* (2013) and named this section *Alternaria* section *alternata*.

The framework described by Lawrence *et al.* (2013) and Woudenberg *et al.* (2013) may lead to difficulties if no species within the *A.* section *Alternaria* can be designated as *Alternaria alternata*. The binomial name *A. alternata* is used in plant pathology, medicine, research into human disease and environmental studies to describe this taxonomically complex group of organisms. Revising the genus in such a way that risks losing this name may cause confusion in the wider scientific and medical community. All isolates in the *A. alternata* species group have an identical ITS sequence (Table 3.1) (Kusaba and Tsuge, 1995b, Pryor and Michailides, 2002). It is logical to assign the ITS genotype as the species *A. alternata* and any distinct lineage within this species (based on highly variable gene regions) as a sub-species of *A. alternata*.

Identification of phylogenetic Clades 1 and 2 as *A. alternata* ssp. *arborescens* and *A. alternata* ssp. *tenuissima* as sub species of *A. alternata* is appropriate. In many studies where morphological differences between taxa have not been observed, authors have called for morphological species names to be abandoned and that these morphological species should be referred to as *A. alternata* (Nishimura, 1980, Nishimura and Kohmoto, 1983, Kusaba and Tsuge, 1994, Kusaba and Tsuge, 1995b, Andrew *et al.*, 2009).

Support from previous studies

Andrew *et al.* (2009) attempted to identify associations between morphological species and phylogenetic clade across 150 *A. alternata* species group isolates. Isolates were identified as having either *A. alternata*, *A. tenuissima* or *A. arborescens* morphologies, or where morphologies could not be identified as having "intermediate" morphologies between morphological species. Phylogenetic clades identified by highly variable loci (including Endopolygalacturonase) showed the presence of multiple morphological species in clades.

This highlights a problem of using species descriptions to identify isolates when morphological characters overlap. This is the advantage of using clustering methods such as applied in this study and those described above, where trends in morphological characters are associated to clades. Despite this weakness in their methodology, Andrew *et al.* (2009) reported no support for differentiation between *A. alternata* and *A. tenuissima* morphological species and reported association between *A. arborescens* morphologies and particular phylogenetic clades (Chapter 3, Fig. 3.5: *A. arborescens* morphology was associated with clade 4; *A. tenuissima* / *A. alternata* morphologies with clades 3 and 5), supporting the findings of this study.

Other studies have also attempted to compare morphological species to molecular phylogenies. Rather than determining a single isolate to be one particular species and comparing that result to phylogenies they have used many measurements of morphological characters or metabolite production profiles to assist phylogenetic identification of lineages. Similar to the work performed in this study they have used clustering methods such as principal components analysis and dendrogram construction to identify groups of similar individuals.

An investigation into the taxonomy of *Alternaria infectoria* species group used a similar approach to recognise species concepts to that performed in this study (Andersen *et al.*, 2009). Phylogenetics was performed on 39 *A. infectoria* isolates and another 12 isolates from related taxa. GSR supported the hypothesis that *A. infectoria* was distinct from related species (*Alternaria malorum*, *Alternaria cetera* and *Embellisia abundans*) but did not support the Simmons (2007) morphological framework of there being 30 species in the *A. infectoria* species group. These findings were supported by clustering analysis using metabolite profiles. High performance liquid chromatography (HPLC) was performed on metabolites and the data from these readings was used to form a Euclidean correlation matrix from which a dendrogram was determined. Their data also showed differences in clustering between the *A. infectoria* group and other species but showed continuous variation within *A. infectoria* isolates. Plotting of the first two principal components was used to display the data in their analysis but was not used to identify informative "peaks" in HPLC metabolite profiles to identify the basis of differentiation between *A. infectoria* isolates and non- *A. infectoria* isolates, as was performed with morphological data in this study.

Alternaria are causal agents of dry core rot of apples in South Africa. A study isolated *A. infectoria* species group and *A. alternaria* species group isolates from mouldy apples and these were identified by ITS sequence data (Serdani *et al.*, 2002). Data on conidial morphology was collected for each isolate, along with metabolite profiles by HPLC. A Euclidean matrix was constructed for this data and a dendrogram constructed. This showed separation of *A. infectoria* from the *A. alternata* species group in support of the ITS data (Serdani *et al.*, 2002). Authors also identified two clades within the *A. alternata* species group, which they believed to represent *A. arborescens* and *A. tenuissima* on the basis of colony characteristics (Fig. 4.12).

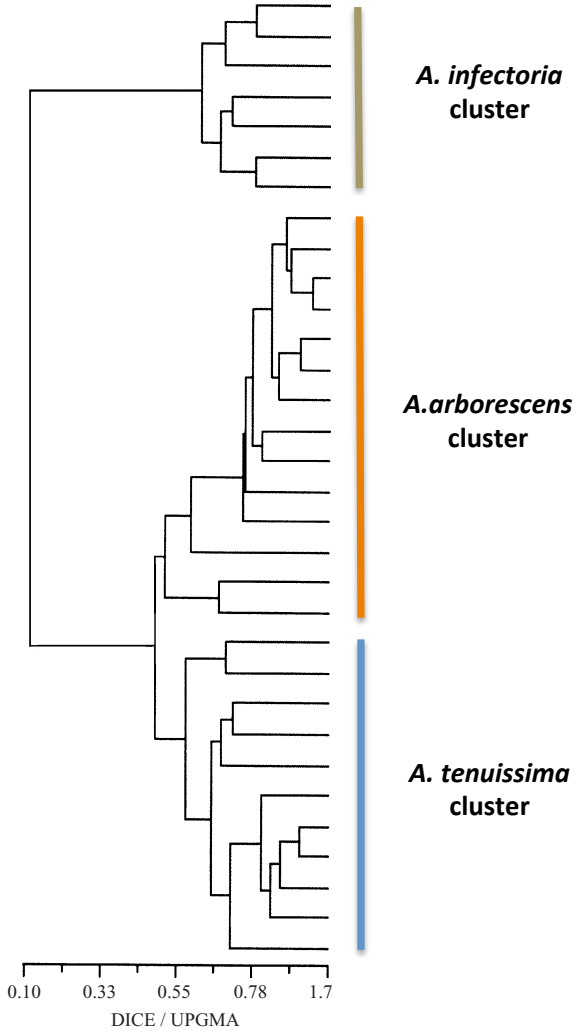


Figure 4.12 UPGMA dendrogram reporting separation of *Alternaria arborescens* and *Alternaria tenuissima* clades: Dendrogram of *Alternaria* isolates causing dry core on apple determined from conidial and morphological characteristics, lesion type on fruit and secondary metabolite produced. Image adapted from Serdani *et al.* (2002).

A recent study investigated the phylogenetic relationships of *A. alternata* species group isolates from apple leaves in Italy (Rotondo *et al.*, 2012). Phylogenies constructed from multi-locus sequence data, AFLP, and inter simple spacer repeats identified either two or three distinct phylogenetic clades. Morphological analysis was performed to test support of phylogenetic groups. Multivariate data was collected as performed in this study, but different spore colony characteristics were used. A dendrogram was constructed using a Euclidian correlation matrix and the resulting tree supported phylogenetic separation of a lineage that could be identified as having an *A. arborescens* morphology from one or two, closely related clades containing mixed *A. alternata* / *A. tenuissima* morphologies (Fig. 4.13).

Using morphological data for automated spore ID

Alternaria sporulation structures have been modelled to predict 3D images of representative spores and sporophores (Schlecht *et al.*, 2007, Taralova *et al.*, 2011): Generalised linear models are used to describe the morphology of *A. alternata*, *A. gaisen*, *A. tenuissima* and *A. arborescens* morphological species as described in Simmons (2007). These models were developed with the aim of performing automated identification. Data collected in this study, and those described above, could be used to validate and refine these models, including testing how often isolates with morphologies similar to *A. alternata* occur. Recent work on *Scopulariopsis brevicaulis* has shown that programs such as Image J (Abramoff *et al.*, 2004), can be used to automatically recognise and count fungal spores within a set size range (Wagner and Macher, 2012). Models such as these may allow automated monitoring of spore levels in air samples, which could be used to improve management of fungal plant pathogens (Wagner and Macher, 2012).

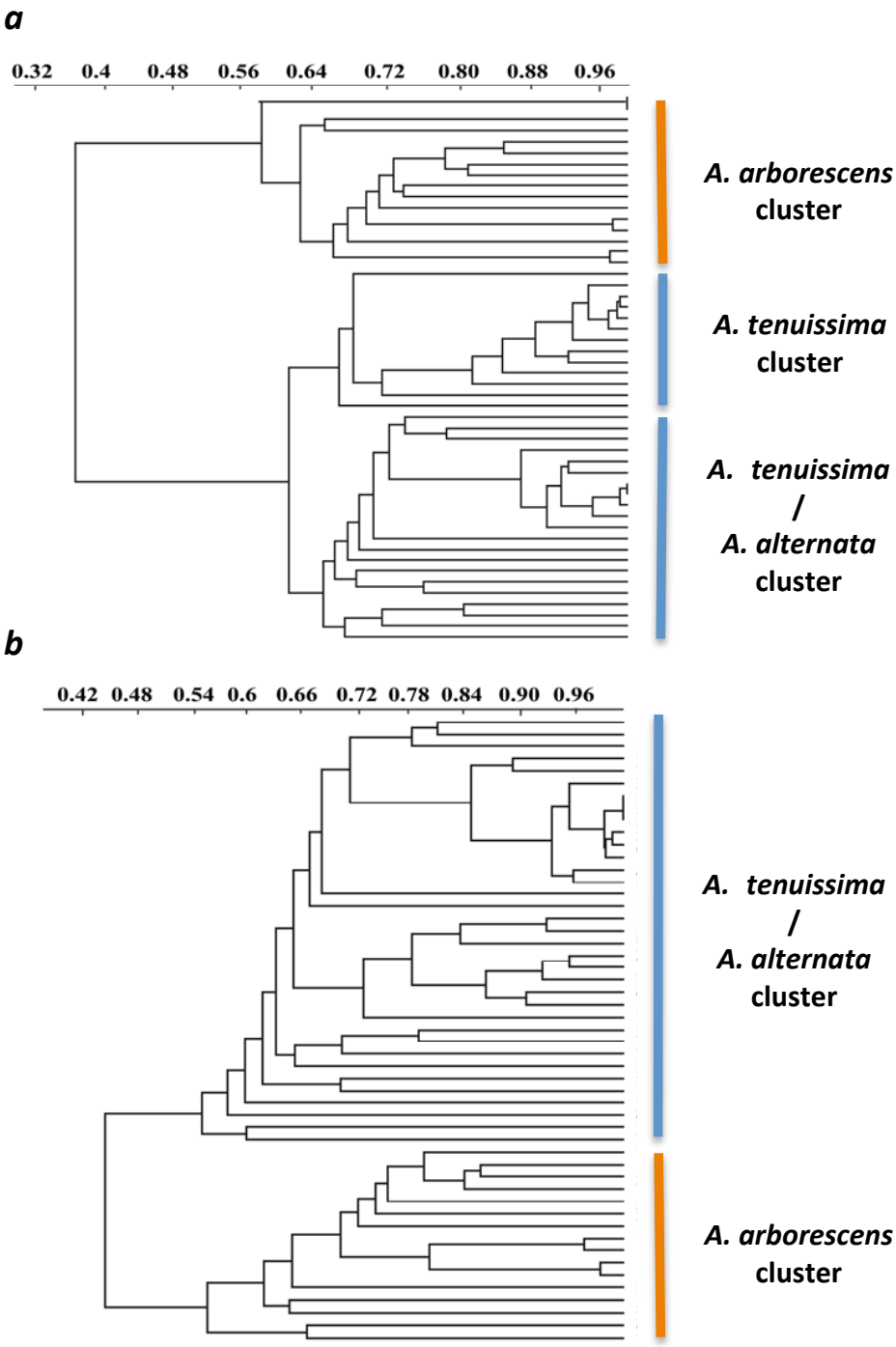


Figure 4.13 AFLP and ISSR phylogenies of *Alternaria* spp. isolates causing leaf spot of apples: *a*) Amplified fragment length polymorphism (AFLP) and *b*) inter simple sequence repeat (ISSR) dendrograms of *Alternaria* spp. isolates causing leaf spot diseases of apple in Italy. Clear separation is seen between *A. arborescens* and *A. tenuissima* clades. Image taken from (Rotondo *et al.*, 2012).

CHAPTER 5

PRESENCE OF TOXIN GENES IN *ALTERNARIA ALTERNATA* ISOLATES

5.1 INTRODUCTION

Alternaria spp. produce host-selective toxins

An acquisition of factors allowing pathogenicity on particular hosts has been suggested to have led the transition in *Alternaria alternata* from a saprophytic to a plant pathogenic lifestyle (Lawrence *et al.*, 2008). These factors are referred to as being "host-specific" or "host-selective" on account of their providing specific pathogenicity to a particular host or range of hosts. The term "host-selective toxin" (HST) is typically used as these toxins may confer pathogenicity to a range of hosts (Maekawa *et al.*, 1984, Itoh *et al.*, 1993).

Host-selective pathogenicity in *A. alternata* is determined by the production of host selective toxins (HSTs). Tsuge *et al.* (2013) summarises previous definitions of a HST as *"a compound that possesses the following characteristics: (1) host-selective toxicity, (2) selective toxicity matching the specificity of the HST-producing pathogen, (3) plants insensitive to the HST must be resistant to the pathogen producing the compound, (4) the compound can reproduce the initial physiological changes in host cells caused by the HST-producing pathogen and (6) the initial physiological changes caused by HST in host cells leads to penetration or initial colonisation by the HST-producing pathogen."* These criteria have been met for eleven taxa within the *Alternaria* genus (Table 5.1) (Gilchrist and Grogan, 1976, Kohmoto *et al.*, 1977, Kohmoto *et al.*, 1979, Maekawa *et al.*, 1984, Otani *et al.*, 1985, Bains and Tewari, 1987, Kodama *et al.*, 1990, Kohmoto *et al.*, 1993, Nutsugah *et al.*, 1994, Otani *et al.*, 1998, Quayyum *et al.*, 2003).

Purified samples of three of the *Alternaria* HSTs (Table 5.1, Toxins: ABT, Destruxin B and APT) have been shown to lose toxicity when heated or by addition of proteinase K, indicating that they are proteinacious (Bains and Tewari, 1987, Otani *et al.*, 1998). Structures of six HSTs have been identified and show structural similarity to polyketides (Tsuge *et al.*, 2013) (Table 5.1). This is supported through the rough lemon toxin (ACRT) being demonstrated to require the presence of a specific polyketide synthase gene for its production (Izumi *et al.*, 2012).

Table 5.1 Host-selective toxins produced by *Alternaria* spp. pathogens causing plant disease.

Pathogen (morphological species name)	Disease	Toxin	Structure
A. alternata apple pathotype (*A. mali*)	*Alternaria* blotch of apple	AMT	Polyketide
A. alternata pear pathotype (*A. gaisen*)	Black spot of Asian pear	AKT	Polyketide
A. alternata rough lemon pathotype (*A. citri*)	Brown spot of rough lemon	ACRT	Polyketide
A. alternata tangerine pathotype (*A. tangelonis*)	Brown spot of tangerine	ACTT	Polyketide
A. alternata strawberry pathotype	*Alternaria* black spot of strawberry	AFT	Polyketide
A. alternata tomato pathotype (*A. arborescens*)	*Alternaria* stem canker of tomato	AALT	Polyketide
A. brassicicola	Black leaf spot of *Brassica*	ABT	Protein
A. brassicae	Grey leaf spot of *Brassica*	Destruxin B	Protein
A. panax	*Alternaria* blight of American gingseng	APT	Protein
A. longipes	Brown spot of tobacco	ATT	Unknown
A. tenuissima	Leaf spot of pigeon pea	ATCT	Unknown

Pathotypes in *Alternaria alternata*

The six *Alternaria* HSTs that possess a polyketide structure have been subject to considerable investigation (Nishimura and Kohmoto, 1983, Otani *et al.*, 1995, Lawrence *et al.*, 2008, Tsuge *et al.*, 2013). The strains that produce these toxins have been shown to be within the *Alternaria alternata* species group (Kusaba and Tsuge, 1995a). These taxa show similarities in moiety between the toxins they produce and may require similar cellular machinery for their synthesis.

The structure of the six polyketide toxins identified in the *A. alternata* species group is unique for each pathotype (Fig. 5.1). However, there are similarities between the structure of pear (AKT) and strawberry (AFT) and tangerine (ACTT) toxins with each containing a 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid moiety (Fig. 5.1). The toxin produced by the apple pathotype (AMT) does not contain this moiety and is primarily cyclic in structure. Each toxin has a number of derivatives that are commonly represented as "R-groups" attached to the common structure for a particular toxin.

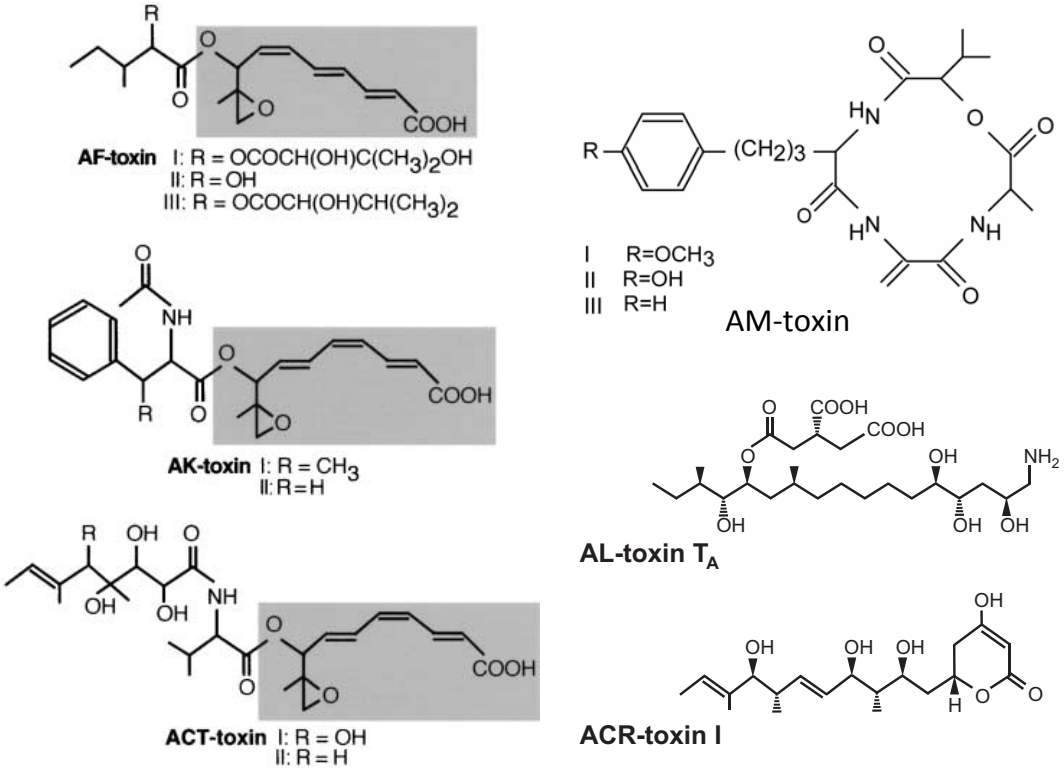


Figure 5.1 Structure of toxins produced by *Alternaria alternata* pathotypes. Structures are displayed for the strawberry (AF), pear (AK) and tangerine (ACT) toxins with the region of common moiety highlighted. Apple (AM), tomato (AL) and rough lemon (ACR) toxin structures are also presented. R-groups display the structural derivatives of toxins (not shown for AL-toxin or ACR-toxin). Images adapted from Hatta *et al.* (2002), Harimoto *et al.* (2007) and Tsuge *et al.* (2013).

A general mechanism of action was proposed for HSTs by Tsuge *et al.* (2013). HSTs are produced by germinating conidia and through their action allow spores to recognise a host; HSTs bind to host receptors leading to suppression of host defences against fungal penetration and aid cellular penetration. In resistant hosts this may lead to induction of defence responses (Fig. 5.2). Tsuge *et al.* (2013) considered HSTs to act as effectors that cause necrotrophic pathogenicity in toxin sensitive plants and that elicit defence in toxin insensitive plants.

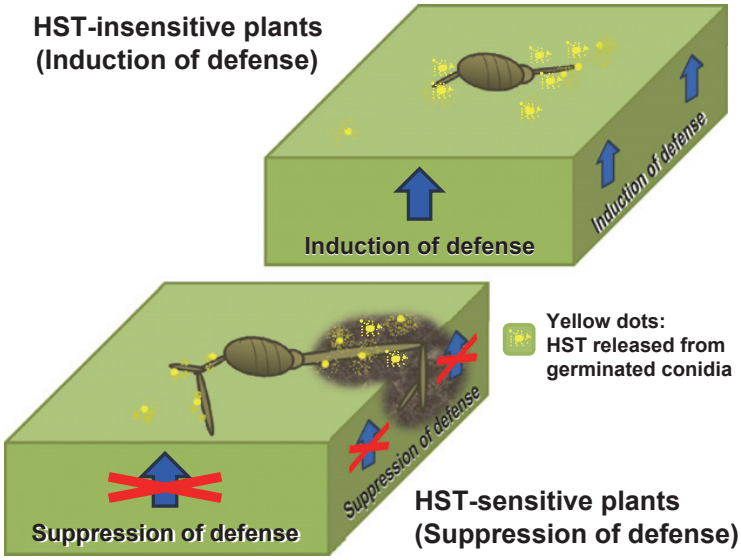


Figure 5.2 *Alternaria* host selective toxins (HSTs) working as effectors: HSTs have suppressor functions to HST-sensitive plants as well as elicitor functions in HST-insensitive plants. Image taken from Tsuge *et al.* (2013).

The specific activities of the toxins produced by the six *A. alternata* pathotypes on host leaves have been investigated. Toxins interact with specific cell membrane receptors causing invagination of plasma membranes and electrolyte leakage in host cells (Ohtani *et al.*, 2002). This aids penetration and initial colonisation of the host and leads directly to necrosis. The pear, strawberry and tangerine toxins have been proposed to have a common mode of action, whereas the rough lemon and apple toxins may have different cellular targets, but still cause leaf necrosis (Fig. 5.3; Otani *et al.* (1995)).

Differences in toxin mechanism have been identified from effects of light exposure. The rough lemon (ACRT) and apple (AMT) toxins have been shown to induce smaller lesions when infections are exposed to light (Tabira *et al.*, 1989, Otani *et al.*, 1995). The apple pathotype only induces necrosis in green tissue suggesting an interaction with the chloroplast (Tabira *et al.*, 1989). Host penetration still occurs when AMT is inoculated with non-pathogenic *A. alternata* spores indicating that AMT has two roles, one of necrosis and another of aiding host penetration (Tabira *et al.*, 1989).

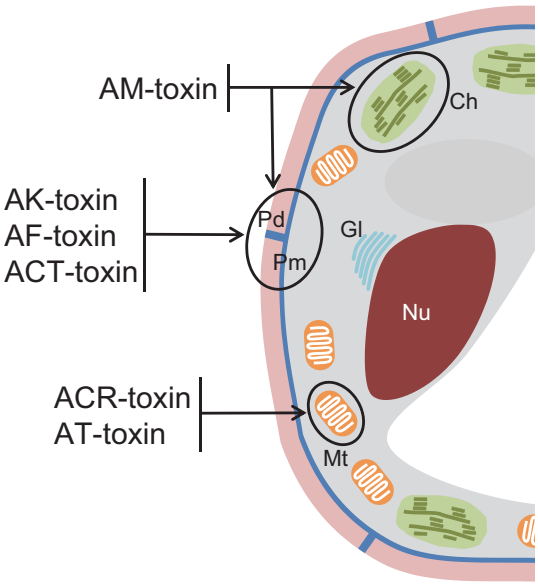


Figure 5.3 Sites of action for toxins produced by *Alternaria alternata* pathotypes. Target sites for apple (AM), pear (AK), strawberry (AF), tangerine (ACT), rough lemon, ACR and tobacco (AT) toxins on cellular components: chloroplasts (Ch), Golgi apparatus (Gl), mitochondrion (Mt), nucleus (Nu) plasmodesmata (Pd) and plasma membrane (Pm). Adapted from Tsuge *et al.* (2013)

The toxicity of an *A. alternata* pathotype toxin is not restricted to the designated host of that pathotype. All or some of the derivatives of a toxin may induce necrosis on "non-target" leaves. For example, AMT from the apple pathotype can induce necrosis on the leaves of Asian pear (Kohmoto *et al.*, 1976).

The genetic basis of host susceptibility to toxins has been investigated. Saito and Takeda (1984) reported that susceptibility to apple HST is determined by a single dominant allele and that resistance is present in apple cultivars that are homozygous recessive for this allele. Sensitivity to the pear HST and the strawberry HST by their respective hosts is controlled by single genes with homozygotes and heterozygotes of the dominant allele conferring susceptibility (Kozaki, 1973, Yamamoto *et al.*, 1985).

The role of conditionally dispensable chromosomes

The polyketide synthase genes responsible for the production of six *A. alternata* species group toxins are present in clusters. Some genes within these clusters have shown homology between pathotypes (Hatta *et al.*, 2002, Miyamoto *et al.*, 2009),

whereas genes are also present within these clusters that are unique to particular pathotypes (Ajiro *et al.*, 2010, Miyamoto *et al.*, 2010). Toxin gene clusters are present on conditionally dispensable chromosomes (CDCs) (Hatta *et al.*, 2002) and studies using pulsed gel field electrophoresis have shown these chromosomes to be approximately 1-2 Mb in size (Table 5.2; Akamatsu *et al.* (1999), Hatta *et al.* (2002), Masunaka *et al.* (2005), Ito *et al.* (2004), Johnson *et al.* (2001)).

Table 5.2 Estimated sizes of conditionally dispensable chromosomes (CDCs) in *Alternaria alternata* pathotypes. Size of CDCs as estimated from pulsed field gel electrophoresis studies. Table adapted from Tsuge *et al.* (2013).

Pathogen	Genes	CDC size
Apple pathotype	*AMT* genes	< 1.8 Mb
Strawberry pathotype	*AFT* genes	1.0 Mb
Pear pathotype	*AKT* genes	< 2.0 Mb
Tangerine pathotype	*ACTT* genes	< 2.0 Mb
Rough lemon pathotype	*ACRT* genes	< 1.5 Mb
Tomato pathotype	*ALT* genes	1.0 Mb

Horizontal gene transfer (HGT) is a potential mechanism for the original acquisition of CDCs and as a mechanism of exchange of CDCs between individuals within *A. alternata*. There is increasing recognition of horizontal gene transfer as a mechanism in the evolution of pathogenicity in fungi (Rosewich and Kistler, 2000, Mehrabi *et al.*, 2011, Richards *et al.*, 2011). Potential for HGT has been demonstrated within the *A. alternata* through protoplast fusion experiments (Salamiah *et al.*, 2001, San Mauro and Agorreta, 2010). Transformants have been made between apple and tomato pathotypes (Salamiah *et al.*, 2001), and between strawberry and tomato pathotypes (San Mauro and Agorreta, 2010) resulting in mixed pathotypes that produced HSTs with the host ranges of both parental strains.

Aside from protoplast fusion, HGT has not been induced in in the lab under what can be considered "natural conditions" (Akagi *et al.*, 2009). The best evidence for HGT occurring naturally has been the isolation of a mixed-pathotype isolate of tangerine and rough-lemon pathotype from a mixed citrus grove in Florida (USA) (Masunaka *et*

al., 2005). This isolate produces both the rough-lemon HST and the tangerine HST and carries two CDCs, one associated with each host. Anastomosis between hyphae of two parental strains, representing two different pathotypes, has been speculated as the origin of this strain (Masunaka *et al.*, 2005). The occurrence of strains that are of mixed-pathotype has not been investigated in any other study.

Structure of conditionally dispensable chromosomes

The genetic database Genbank contains sequence data from toxin gene clusters of a number of *A. alternata* pathotypes. Studies making use of bacterial artificial chromosomes (BAC) have led to the sequencing of toxin gene cluster regions from three *A. alternata* apple pathotype isolates (Genbank accessions: AB525198, AB525199, AB525200; Unpublished). These sequences are 100-130 Kb in size and contain 17 genes that are considered to be involved in synthesis of the apple toxin (Fig. 5.4; Harimoto *et al.* (2007)). *AMT1*, *AMT2*, *AMT3* and *AMT4* have been demonstrated to be involved in AMT synthesis, as gene disruption experiments have led to reduction in AMT production (Johnson *et al.*, 2000a, Harimoto *et al.*, 2007, Harimoto *et al.*, 2008). Experimental evidence has not been provided to show that the remaining 13 *AMT* genes, as annotated to the Genbank accessions above, have a role in toxin production. Four genes present in the CDC for the pear pathotype have been sequenced and have been named *AKT1*, *AKT2*, *AKT3*, *AKTR-1* (Tanaka *et al.*, 1999, Tanaka and Tsuge, 2000, Tsuge *et al.*, 2013) and a further two genes (*AKT4*, *AKTS1*) have been reported but have not been published (Tsuge *et al.*, 2013). Regions of toxin gene clusters have been sequenced from BAC clones for the *A. alternata* strawberry pathotype (17 Kb region), which contained six (of the 10 currently identified) *AFT* genes (Hatta *et al.*, 2002, Ruswandi *et al.*, 2005, Hatta *et al.*, 2006), and the tangerine pathotype (33 Kb region), which contained six genes that have been shown to be associated with ACTT production: *ACTT1*, *ACT2*, *ACTT3*, *ACTT4*, *ACTT5* and *ACTT6* (Masunaka *et al.*, 2000, Masunaka *et al.*, 2005, Miyamoto *et al.*, 2008, Miyamoto *et al.*, 2009, Miyamoto *et al.*, 2010). The sequence of one rough lemon gene (*ACRTS1*) has been published (Izumi *et al.*, 2012), and sequence data is publically available for a second (*ACRTS2*) but is currently unpublished. A single

gene has been identified in ALT synthesis in the tomato pathotype (*ALT1*) and is publically available (Akagi *et al.*, 2009). Recent sequencing of the tomato pathotype by Hu *et al.* (2012) has led to the identification of contigs that do not align to the *A. brassicicola* genome and from this up to 200 genes have been identified that could be present on the tomato pathotypes CDC.

Genes on CDCs in toxin-gene clusters have been reported as being unique to particular pathotypes, such as *AMT1* in the apple pathotype (Johnson *et al.*, 2000a), and *AKTS1* in the pear pathotype (Tsuge *et al.*, 2013). However a number of genes within toxin-gene clusters show homology between different pathotypes. DNA probes of *AKT1* and *AKT2* have been shown to hybridise to regions within digested genomes of the strawberry pathotype and the tangerine pathotype (Tanaka *et al.*, 1999). Genes in the pear and tangerine pathotypes were shown to be homologous to one another by Masunaka *et al.* (2000); Sequencing and alignment showed that *AKT1* and *ACTT1* were homologous and that *AKT2* and *ACTT2* genes were also homologs of each other. Unpublished work has reported homology between the pear, strawberry and citrus pathotypes for all *AKT*-genes apart from *AKTS1* (Tsuge *et al.*, 2013). The apple pathotype produces AMT, which is structurally different to other HSTs, but this has also been shown to share common genes with other pathotypes. The *AMT2* gene was shown by Ito *et al.* (2004) to be homologous to a toxin gene in the strawberry pathotype (*AFTS1*). As HSTs are polyketides derived from secondary metabolism homologous genes between pathotypes suggest that common "cellular machinery" is used in their synthesis. The homologous genes in pear, strawberry and tangerine toxin-gene clusters are thought to be responsible for the common a 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid moiety in their respective toxins (Tsuge *et al.*, 2013).

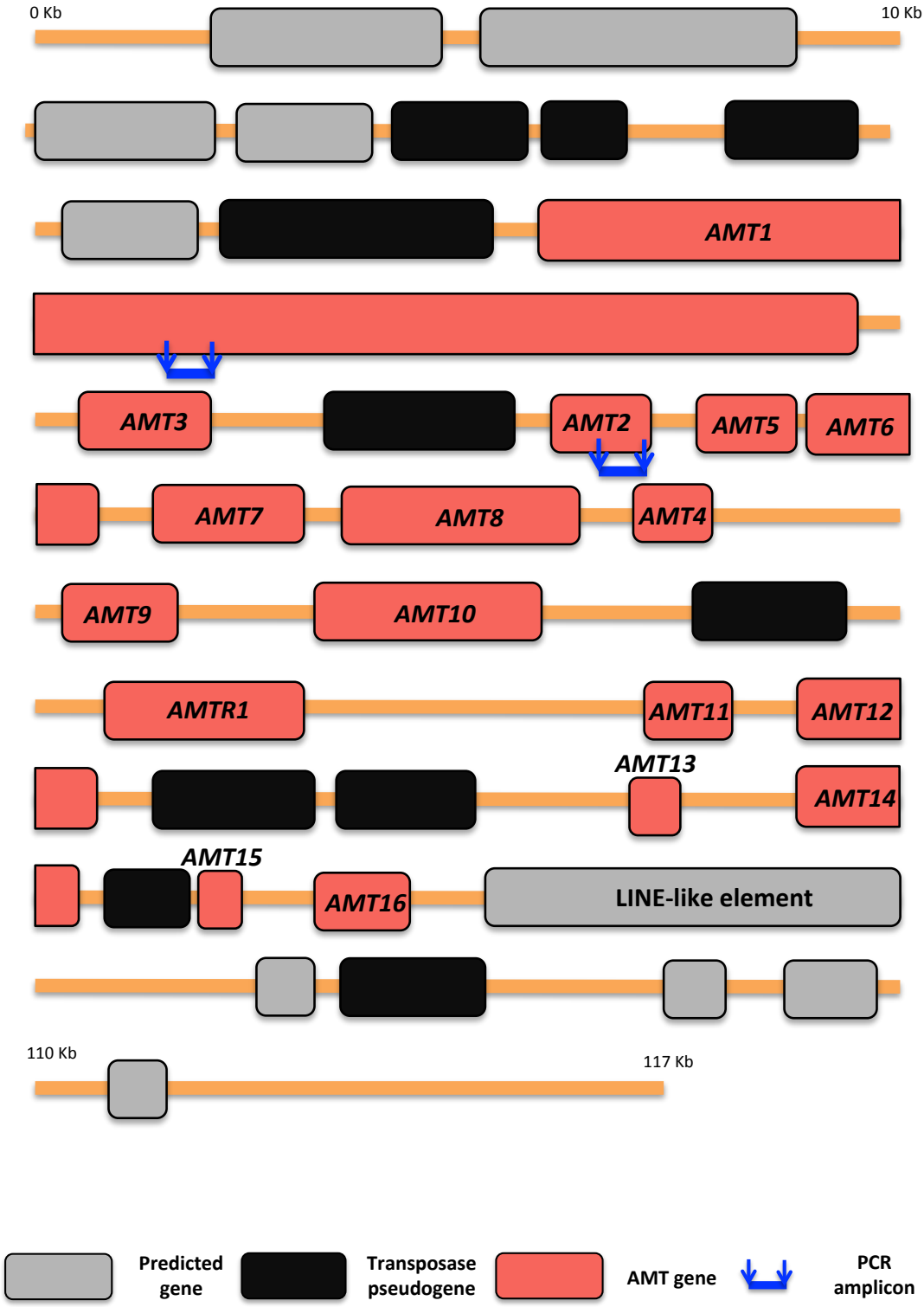


Figure 5.4 Toxin gene cluster in an *Alternaria alternata* apple pathotype isolate: Genetic sequence and annotation as presented for Genbank accession: AB525198.1. The 117 Kb sequence contains 17 genes considered to be involved in AMT synthesis. Other predicted genes are shown including a "Line-like" transposon. The target amplicon for primers designed to *AMT1* and *AMT2* regions are shown.

Genetic rearrangement may have played a large part in the evolution of CDCs, potentially through the action of transposable elements. Five transposons have been identified on the 17 Kb CDC region of the *A. alternata* strawberry pathotype (Hatta *et al.*, 2006). These were identified as being inactive due to deletions, termination codons and frame-shifts. They were therefore determined to be pseudogenes and were considered as "transposon fossils" (Hatta *et al.*, 2006). BAC clones for the AMT toxin gene regions also show the presence of transposable elements. A 117 Kb region from the Genbank accession AB525198.1 contains 10 transposase pseudogenes and a transposon gene that annotation has identified as having a long interspersed element (LINE) -like element (Fig. 5.4). LINEs are genetic elements that code for reverse transcriptase, allowing their reintegration into the genome once transcribed into RNA (Singer, 1982). Dispersed repeat sequences such as transposable elements have been determined to serve as sites of recombination within and between fungal chromosomes (Zolan, 1995).

PCR primers have been designed for genetic regions within *A. alternata* pathotype gene clusters. Primers have been designed for two of the 16 apple toxin gene regions (Fig. 5.4), one within the *AMT1* gene region (Johnson *et al.*, 2000b) and the second within the *AMT2* gene region (Roberts *et al.* (2011); Fig. 5.4). The *AMT1* gene encodes a cyclic peptide synthase protein that is specific to the *A. alternata* apple pathotype (Johnson *et al.*, 2000a). PCR primers have also been designed for two toxin genes contained within the pear toxin gene cluster *AKT1* and *AKT2* (Roberts *et al.*, 2011). Primer sets for *AMT* genes and *AKT* genes were designed to allow identification of apple and pear pathotypes (Johnson *et al.*, 2000b, Roberts *et al.*, 2011).

Phylogenetic distribution of toxin-synthesis genes

Phylogenetic studies have attempted to determine whether *A. alternata* pathotypes containing specific toxin genes are distinct phylogenetic taxa. The ITS sequence is identical for all pathotypes (Kusaba and Tsuge, 1995a) leading to these isolates all being designated as *A. alternata*. With the identification of highly variable molecular

markers, studies can be performed investigating the distribution of isolates carrying toxin genes within *A. alternata* species group phylogenies.

Results of phylogenetics of the *A. alternata* pathotypes could be used to inform diagnostics. If genes on essential chromosomes can be identified that can phylogenetically resolve the different *A. alternata* pathotypes then these could act as molecular markers. QBOL, a recent initiative to barcode and develop molecular diagnostics for quarantine pathogens in the EU is based upon this approach (Bonants *et al.*, 2010, Quaedvlieg *et al.*, 2012). Establishing whether identification can be performed based on sequencing of phylogenetic loci, or the use of toxin-gene specific primers, is of particular importance for the *A. alternata* apple pathotype and the *A. alternata* pear pathotype due the phytosanitary risk posed by their potential establishment in the UK and Europe.

5.2 AIMS

The diversity, structure and expression of *Alternaria* toxins are relatively well understood. There has also been some work investigating the occurrence and distribution of genes involved in toxin synthesis within the *A. alternata* species group. This research has been based upon low variability phylogenetic loci or restricted to a particular pathosystem. As a result the evolution of the different *A. alternata* pathotypes is poorly understood. The aim of this chapter was to investigate the distribution of toxin genes within the multi-locus phylogeny of *A. alternata* species group determined in Chapter 3. This involved using PCR to screen large numbers of *Alternaria* isolates for a limited number of toxin genes and also involved screening genome sequence data from a subset of isolates for all (publicly available) *Alternaria* toxin genes. Requirement of these toxin genes for pathogenicity was also investigated.

Specific aims were as follows:

1. Screen 115 *A. alternata* species group isolates for the presence of two apple and two pear toxin genes and identify their distribution throughout the multi-locus phylogeny determined in Chapter 3 (Fig. 3.2).
2. Search the genomes of 12 *A. alternata* strains for the presence of 40 genes known to be involved host-selective toxin synthesis
3. Test whether *A. alternata* isolates containing genes required for apple toxin (AMT) synthesis cause more leaf lesions on apple leaves than isolates without these genes.

5.3 METHODS

PCR screens for two apple and two pear toxin-synthesis genes

A. alternata isolates (115) from the University of Warwick culture collection were screened for the presence of two genes involved in apple toxin production (*AMT1* and *AMT2*) and two genes involved in pear toxin gene production (*AKT1* and *AKT2*). PCR was performed for each of these four loci separately using published primers and reaction conditions described in Chapter 2 (Table 2.2). PCR products were visualised using gel electrophoresis and amplicon size estimated using a DNA ladder (Invitrogen 1 Kb Plus DNA Ladder; including fragment sizes at 100, 200, 300, 400, 500, 650, 850, 1000 bp). PCR products from a number of isolates that showed a single electrophoresis band at the expected size (458 bp, 613 bp, 310 bp or 514bp for *AMT1*, *AMT2*, *AKT1* or *AKT2* respectively) were confirmed to represent the target gene through Sanger sequencing (Chapter 2). This was performed for four isolates for *AMT1*, four isolates for *AMT2*, three isolates for *AKT1* and three isolates for *AKT2*. Sequence data was confirmed to be *AMT1*, *AMT2* by comparing sequence data to the *AMT* gene cluster sequence reported in Harimoto *et al.* (2007) (Genbank accession: AB525198.1); or the *AKT1* and *AKT2* gene regions by comparison to published *AKT* gene regions (Genbank accessions: AB015351 and AB015352, respectively; reported in Tanaka *et al.* (1999)). Presence of toxin genes was then indicated on the multi-locus phylogeny constructed in Chapter 3 (Fig. 3.2).

BLAST searches of 40 toxin synthesis genes against *Alternaria alternata* genomes

Sequence data for 40 genes involved in *A. alternata* HST synthesis were downloaded from Genbank (Table 5.3). This included 16 genes from the apple pathotype, four genes from the pear pathotype, 10 genes from the strawberry pathotype, six genes from the tangerine pathotype, two genes from the rough lemon pathotype and one gene from the tomato pathotype.

Geneious (Kearse *et al.*, 2012) was used to perform BLASTn searches of all 40 toxin gene sequences against one another to identify whether genes in this list were homologs of one another. The alignment-length, e-value and number of identical sites were recorded for each BLASTn result.

The *de novo* genome assemblies of 12 *A. alternata* strains (Table 2.3) were imported into Geneious (Kearse *et al.*, 2012) where a BLAST database was made for each assembly. BLASTn was used to search for the presence of 40 *A. alternata* toxin genes (Table 5.3) in these genomic databases. The single best alignment was returned for each toxin gene, with a minimum e-value of $1x10^{-10}$. The alignments length, e-value, and number of identical sites were recorded for each BLAST alignment. Details were also recorded for each contig an alignment was made to, including the length of that contig and its mean coverage by sequence data during genome assembly (as determined by Velvet; Zerbino and Birney (2008)). To identify whether toxin genes were in multiple copies in the genome, the coverage of contigs containing alignments was compared to the mean coverage of all contigs in the N50 (Table 2.3). Alignments were inspected visually in Geneious as appropriate.

Table 5.3 Toxin genes used in BLAST searches against *Alternaria alternata* genome sequence: Sequence length, Genbank accession numbers and source of sequence data for 40 publicly available genes involved in toxin synthesis for six *A. alternata* pathotypes.

Pathotype	Gene	Length (bp)	Genbank Accession no.	Published in
Apple	***AMT1***	13092	AB525198.1	Harimoto *et al.* (2007)
	AMT2	1254	AB525198.1	Harimoto *et al.* (2007)
	AMT3	1561	AB525198.1	Harimoto *et al.* (2007)
	AMT4	913	AB525198.1	Harimoto *et al.* (2007)
	AMT5	1223	AB525198.1	Harimoto *et al.* (2007)
	AMT6	1403	AB525198.1	Harimoto *et al.* (2007)
	AMT7	1797	AB525198.1	Harimoto *et al.* (2007)
	AMT8	2829	AB525198.1	Harimoto *et al.* (2007)
	AMT9	1411	AB525198.1	Harimoto *et al.* (2007)
	AMT10	2703	AB525198.1	Harimoto *et al.* (2007)
	AMT11	1008	AB525198.1	Harimoto *et al.* (2007)
	AMT12	1248	AB525198.1	Harimoto *et al.* (2007)
	AMT13	497	AB525198.1	Harimoto *et al.* (2007)
	AMT14	1105	AB525198.1	Harimoto *et al.* (2007)
	AMT15	555	AB525198.1	Harimoto *et al.* (2007)
	AMT16	1183	AB525198.1	Harimoto *et al.* (2007)
	AMTR1	2461	AB525198.1	Harimoto *et al.* (2007)
Pear	***AKT1***	2028	AB015351	Tanaka *et al.* (1999)
	AKT2	849	AB015352	Tanaka *et al.* (1999)
	AKT3	1061	AB035492	Tanaka and Tsuge (2000)
	AKTR	1335	AB035491	Tanaka and Tsuge (2000)
Strawberry	***AFT1-1***	2027	AB070711	Hatta *et al.* (2002)
	AFT3-1	1074	AB070713	Hatta *et al.* (2002)
	AFT3-2	1075	AB179766	Ruswandi *et al.* (2005)
	AFT9-1	9073	AB179766	Ruswandi *et al.* (2005)
	AFT10-1	1843	AB179766	Ruswandi *et al.* (2005)
	AFT11-1	1746	AB179766	Ruswandi *et al.* (2005)
	AFT12-1	894	AB179766	Ruswandi *et al.* (2005)
	AFTS1	1252	AB119280	Ito *et al.* (2004)
	AFTR-1	1338	AB070712	Hatta *et al.* (2002)
	AFTR-2	1335	AB179766	Ruswandi *et al.* (2005)
Tangerine	***ACTT1***	785	AB034586	Masunaka *et al.* (2000)
	ACTT2	849	AB432914	Miyamoto *et al.* (2009)
	ACTT3	1061	AB176941	Miyamoto *et al.* (2009)
	ACTTR	1308	AB176941	Miyamoto *et al.* (2009)
	ACTT5	1883	AB444613	Miyamoto *et al.* (2009)
	ACTT6	897	AB444614	Miyamoto *et al.* (2009)
Rough lemon	***ACRTS1***	1394	AB688098	Izumi *et al.* (2012)
	ACRTS2	7958	AB725683	Unpublished
Tomato	***ALT1***	668	AB4656676	Akagi *et al.* (2009)

Virulence assay for isolates possessing apple toxin-synthesis genes

The hypothesis "Isolates testing positive for *AMT* toxin genes do not cause greater incidence of lesions than isolates that were not *AMT* positive, or water controls" was tested. This was performed in two experiments, one using apple *cv.* Spartan leaves and one using apple *cv.* Bramley's seedling leaves.

Unfolded adult apple leaves, less than 10 cm in length were cut from young (less than 12 months old) apple *cv.* Spartan trees or *cv.* Bramley's seedling trees. These were quality-checked to ensure that they were healthy and free from disease. Leaves were grouped by similar size and age and organised into ten groups of nine leaves. Nine leaves were placed into each of ten 20 cm x 20 cm x 10 cm plastic containers. Leaves were arranged in a 3 x 3 grid in each box, with the abaxial leaf surface facing upwards (Fig. 5.5). The base of these boxes was lined with two sheets of white towel, and wetted with 50 mls of sterile distilled water (SDW). Each of the ten boxes formed one experimental replicate.

Cultures used for pathogenicity assays were from single spore cultures made during the preparation of isolates for genome sequencing (Chapter 2). Spore suspensions were made by growing *A. alternata* isolates on 1% PDA plates (see Chapter 2) for four weeks at 23 °C before flooding the plate with 2 mls of SDW, scraping the plate with a disposable L-shaped spreader. The concentration of spores in the resultant spore suspension was determined using a haemocytometer and adjusted to a concentration of $1x10^5$ spores.ml^{-1}.

Each leaf was either inoculated with 10 μl of *A. alternata* spore suspension or 10 μl of sterile-distilled water at six points on the abaxial leaf surface. Of the nine leaves in each box, three leaves were inoculated with spore suspension from isolates carrying apple toxin genes (*FERA 635*, *743* and *1166*: Table 5.6), three leaves were inoculated with spore suspensions from isolates not testing positive for both apple toxin genes (*FERA 648*, *1082* and *1164*: Table 5.6), and three leaves were inoculated with SDW. A single *A. alternata* isolate was used to infect each leaf. Three isolates whose genomes indicated presence of the apple toxin gene cluster ("*AMT* positive"; *FERA 635*, *743*, *1166*) were inoculated and three isolates whose genomes did not ("AMT negative"; FERA *648*, *1082* and *1164*) were also inoculated. These six isolates were

selected from phylogenetic Clade 2 of the multi-locus phylogeny constructed in Chapter 3 (Fig. 3.2). Following inoculation, each container was sealed and placed in plastic bags to prevent moisture loss. Boxes were then kept in a growth cabinet (Sanyo) held at 23 °C with a 12 hr. light / 12 hr. dark cycle for 28 days. Boxes were removed for imaging at 7 days post inoculation (dpi), 14 dpi, 21 dpi and 28 dpi. Photographs were taken of each box with the lid removed using a digital camera (Lumix FZ45, Panasonic) held in a constant position and at a fixed height of 32 cm. Each of the nine leaves within each image was assessed for presence of necrotic lesions, and the number of lesions was recorded.

Lesions were clearly identifiable at 14 dpi and had not coalesced. Data collected at 14 dpi was used for statistical analysis in both apple *cv.* Spartan, and *cv.* Bramley's seedling datasets. Binomial regression using a generalised linear model (GLM) was used to analyse the number of lesions per leaf. Three terms were included in the model to explain the response variable: The first term in the model was Treatment, which tested whether the effect of the three inoculation treatments in the model were significant (*AMT* positive, *AMT* negative or SDW). A second term was included in the model to test whether the three different *Alternaria* strains within each treatment differed from one another (Isolate). A third term was included to test for any significant effect between each of the ten replicate boxes in the experiment (Box replicate). Residual mean deviance scores were checked to confirm that models showed a good fit to the data.

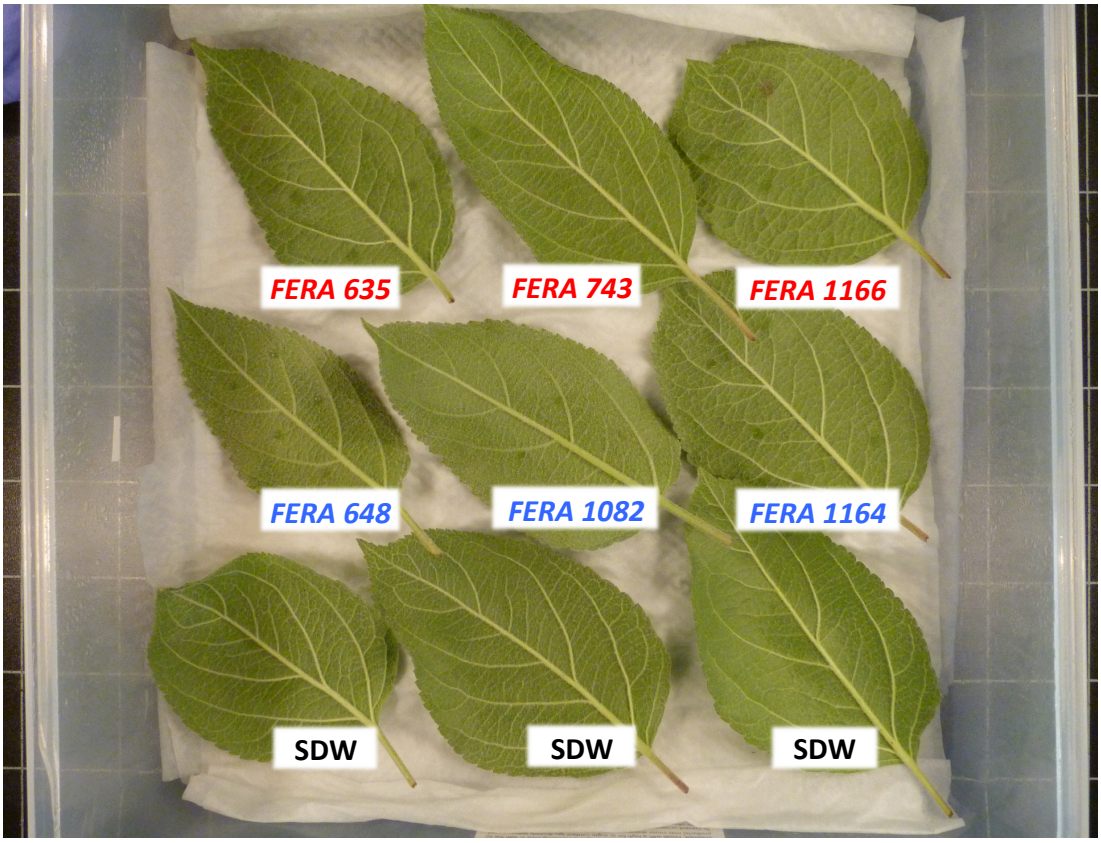


Figure 5.5 Example of a single experimental replicate used in leaf virulence assays: Nine apple *cv.* Spartan leaves inoculated with: Isolates testing positive for *AMT* (top row); Isolates not testing positive for both *AMT* genes (middle row); Sterile distilled water (SDW) controls (bottom row).

5.4 RESULTS

PCR screens for two apple and two pear toxin-synthesis genes

PCR screens for *AMT1*, *AMT2*, *AKT1* and *AKT2* genes displayed secondary banding in electrophoresis gels for a number of samples. Sanger sequencing was performed on a subset of samples to confirm that clear single bands of the expected sizes represented the target amplicon.

Sequence data generated for *AMT1* from four isolates with clear single bands of 458 bp (Table 5.4), showed complete identity to the target regions within the *AMT1* gene in Genbank accession AB525198.1 except for a single SNP at base 360 in the alignment. As such the presence of a single band at 458 bp in gels confirmed the presence of *AMT1*. Sequence data for *AMT2* from four isolates (marked in Table 5.4), showed complete identity to the *AMT2* region in Genbank accession AB525198.1. As such the presence of a single band at 613 bp in gels confirmed presence of *AMT2*. Sequence data for *AKT1* from three isolates, marked in Table 5.4, showed complete identity to the *AKT1* region in Genbank accession AB015351. As such the presence of a single band at 310 bp in gels showed presence of *AKT1*. Sequence data for *AKT2* from three isolates, marked in Table 5.4, showed complete identity to the *AKT2* region in Genbank accession AB015352. As such the presence of a single band at 613bp in gels confirmed presence of *AKT2*.

Amplicons for the *AMT1* gene were identified in 20 of the 115 isolates tested while amplicons for the *AMT2* gene were present in 30 isolates (Table 5.4). 16 isolates were *AMT1+AMT2* positive (Table 5.4). High levels of secondary banding were observed for >50% of isolates (Fig. 5.6:*a*, *b*). Amplicons for the *AKT1* gene were identified in 17 of the 115 isolates tested while amplicons for the *AKT2* gene were present in 12 isolates (Table 5.4). Seven isolates were *AKT1+AKT2* positive (Table 5.4). Secondary banding was present in one lane of *AKT1* and in 18 lanes of *AKT2* (Fig. 5.6:*c*, *d*).

Table 5.4.*a* Toxin gene screening results: Isolates testing positive apple toxin genes (*AMT1* and *AMT2*) and pear toxin genes (*AKT1* and *AKT2*) as determined by banding in gel electrophoresis lanes (Fig. 5.6-Fig. 5.9). Shaded cells mark PCR product that was sequenced to confirm identity to target regions.

Lane no.	Isolate	Host		Clade no.	AMT1	AMT2	AKT1	AKT2
1	*FERA 39*	Schefflera	*Schefflera sp.*					
2	*FERA 156*							
3	*FERA 326*	Asian pear	*Pyrus pyrifolia*	*2a*				
4	*FERA 348*	Apple	*Malus domestica*	*1a*				
5	*FERA 469*	Mandarin orange	*Citrus reticulata*	*2c*				
6	*FERA 478*	Strawberry	*Fragaria sp.*	*1b*				
7	*FERA 479*	Strawberry	*Fragaria sp.*	*1b*				
8	*FERA 482*	Strawberry	*Fragaria sp.*	*2b*				
9	*FERA 483*							
10	*FERA 484*			*1a*				
11	*FERA 488*	Peach	*Prunus persica*	*1a*				
12	*FERA 538*	Asian pear	*Pyrus pyrifolia*	*2a*	√	√		
13	*FERA 540*	Pear	*Pyrus sp.*	*2a*				
14	*FERA 612*	Brussel sprout	*Brassica oleracea*	*14*		√		
15	*FERA 631*	European pear	*Pyrus communis*	*1a*				
16	*FERA 632*	Pear	*Pyrus sp.*	*3*			√	√
17	*FERA 634*	Apple	*Malus domestica*	*2d*			√	
18	*FERA 635*	Apple	*Malus domestica*	*2a*	√	√	√	√
19	*FERA 647*	Pear	*Pyrus sp.*	*1b*			√	
20	*FERA 648*	Pear	*Pyrus sp.*	*2b*	√			
21	*FERA 650*	Pear	*Pyrus sp.*	*3*			√	√
22	*FERA 675*	Pear		*1a*			√	
23	*FERA 678*	Apple	*Malus domestica*	*2a*				
24	*FERA 679*	Apple	*Malus domestica*	*2a*				
25	*FERA 680*	Busy lizzie	*Impatiens walleriana*					
26	*FERA 681*	Pear	*Pyrus sp.*	*1a*				
27	*FERA 704*	Pear	*Pyrus sp.*	*2a*			√	
28	*FERA 743*	Apple	*Malus domestica*	*2a*	√	√		
29	*FERA 800*							
30	*FERA 802*							
31	*FERA 803*							
32	*FERA 805*				√	√	√	√
33	*FERA 833*	Apple	*Malus domestica*	*2a*	√	√		
34	*FERA 840*					√	√	
35	*FERA 1082*	Apple	*Malus domestica*	*2d*		√		
36	*FERA 1105*			*2c*	√	√		
37	*FERA 1164*	Apple	*Malus domestica*	*2e*		√		
38	*FERA 1165*	Apple	*Malus domestica*	*2e*			√	√
39	*FERA 1166*	Apple	*Malus domestica*	*2b*	√	√		√
40	*FERA 1167*	Apple	*Malus domestica*					
41	*FERA 1168*	Apple	*Malus domestica*			√		
42	*FERA 1169*	Apple	*Malus domestica*	*1b*				
43	*FERA 1170*	Apple	*Malus domestica*	*1b*				
44	*FERA 1171*	Apple	*Malus domestica*	*1a*	√			
45	*FERA 1172*	Apple	*Malus domestica*	*1b*				
46	*FERA 1173*	Apple	*Malus domestica*					
47	*FERA 1174*	Apple	*Malus domestica*					
48	*FERA 1175*	Apple	*Malus domestica*					
49	*FERA 1176*	Apple	*Malus domestica*					
50	*FERA 1177*	Apple	*Malus domestica*	*2d*	√	√		√
51	*FERA 1184*	Apple	*Malus domestica*					
52	*FERA 1307*	Carrot	*Daucus carota*					
53	*FERA 1308*	Carrot	*Daucus carota*					
54	*FERA 1310*	Apple	*Malus domestica*	*2d*				
55	*FERA 1410*			*2b*				
56	*FERA 1490*	Strawberry	*Fragaria sp.*	*2b*		√		
57	*FERA 1491*	Strawberry	*Fragaria sp.*	*2d*		√	√	
58	*FERA 1512*	Chrysanthemum	*Dendrathema sp.*	*1b*				

Table 5.4.*b* Toxin gene screening results: Isolates testing positive apple toxin genes (*AMT1* and *AMT2*) and pear toxin genes (*AKT1* and *AKT2*) as determined by banding in gel electrophoresis lanes (Fig. 5.6-Fig. 5.9). Shaded cells mark PCR product that was sequenced to confirm identity to target regions.

Lane no.	Isolate	Host		Clade no.	AMT1	AMT2	AKT1	AKT2
59	*FERA 1513*	Chrysanthemum	*Dendrathema sp.*					
60	*FERA 1518*	Tomato	*Solanum lycopersicum*	*1a*				
61	*FERA 1519*	Cotton	*Gossypium sp.*	*2d*				
62	*FERA 1520*	Lettuce	*Lactuca sp.*	*2d*				
63	*FERA 1521*	Apricot	*Prunus armeniaca*	*1a*				
64	*FERA 1522*	Kiwi	*Actinidia sp.*	*1b*				
65	*FERA 1523*	Watermelon	*Citrullus lanatus*	*2d*		√		√
66	*FERA 1539*	Walnut	*Juglans regia*	*1a*				
67	*FERA 1551*	Apple	*Malus domestica*	*2a*	√	√		
68	*FERA 2038*	Tomato	*Solanum lycopersicum*	*1b*				
69	*FERA 2040*	Apple	*Malus domestica*	*1a*				
70	*FERA 2041*	Apple	*Malus domestica*	*2a*				
71	*FERA 2042*	Apple	*Malus domestica*		√	√		
72	*FERA 2043*	Apple	*Malus domestica*	*2a*				
73	*FERA 2044*	Apple	*Malus domestica*	*2a*				
74	*FERA 2103*	Citrus	*Citrus sp.*	*2b*				
75	*FERA 2277*	Busy lizzie	*Impatiens walleriana*	*1b*				
76	*FERA 2280*	Busy lizzie	*Impatiens walleriana*	*1b*				
77	*FERA 2639*	Brassica	*Brassica sp.*		√	√		√
78	*FERA 2646*	Sweet potato	*Ipomoeaa batatas*					
79	*FERA 2712*	Citrus	*Citrus sp.*	*2c*	√	√	√	√
80	*FERA 3250*			*2d*				
81	*FERA 9205*	Salad rocket	*Eruca vesicana*			√		
82	*FERA 9285*	Busy lizzie	*Impatiens walleriana*	*1b*		√	√	
83	*FERA 15182*	Potato	*Solanum tuberosum*			√	√	
84	*FERA 15968*	Potato	*Solanum tuberosum*	*2d*	√	√		
85	*FERA 20730*				√	√		
86	*FERA 21677*	Pear	*Pyrus sp.*	*2a*	√	√		
87	*FERA 23766*	Asian pear	*Pyrus pyrifolia*	*2b*		√		√
88	*FERA 24350*	Asian pear	*Pyrus pyrifolia*	*2a*	√	√		
89	*EGS 34.015*	Carnation	*Dianthus sp.*	*2b*				
90	*EGS 34.016*	Peanut	*Arachis sp.*	*2c*				
91	*EGS 38.029*	Apple	*Malus domestica*	*2b*				
92	*EGS 90.0512*	Asian pear	*Pyrus pyrifolia*	*3*			√	√
93	*RGR 97.0007*	Apple	*Malus domestica*	*1a*			√	
94	*RGR 97.0008*	Apple	*Malus domestica*	*1a*				
95	*RGR 97.0009*	Apple	*Malus domestica*	*1a*				
96	*RGR 97.0010*	Apple	*Malus domestica*	*1a*				
97	*RGR 97.0011*	Apple	*Malus domestica*	*1a*				
98	*RGR 97.0012*	Apple	*Malus domestica*	*1a*				
99	*RGR 97.0013*	Apple	*Malus domestica*	*1a*				
100	*RGR 97.0014*	Apple	*Malus domestica*	*1a*				
101	*RGR 97.0015*	Apple	*Malus domestica*	*1a*				
102	*RGR 97.0016*	Apple	*Malus domestica*	*1a*			√	
103	*RGR 97.0017*	Apple	*Malus domestica*	*1a*				
104	*RGR 97.0018*	Apple	*Malus domestica*	*1a*				
105	*RGR 97.0019*	Apple	*Malus domestica*	*1a*				
106	*RGR 97.0020*	Apple	*Malus domestica*	*1a*				
107	*RGR 97.0021*	Apple	*Malus domestica*	*1a*				
108	*RGR 97.0022*	Apple	*Malus domestica*	*1a*				
109	*RGR 97.0024*	Apple	*Malus domestica*	*1a*				
110	*RGR 97.0025*	Apple	*Malus domestica*	*1a*				
111	*RGR 97.0026*	Apple	*Malus domestica*	*1a*				
112	*RGR 97.0027*	Apple	*Malus domestica*	*1a*				
113	*RGR 97.0067*	Apple	*Malus domestica*	*1a*				
114	*HA-1*	Apple	*Malus domestica*	*2b*	√	√		
115	*O-159*	Apple	*Malus domestica*	*2b*	√	√		
116	-ve control							

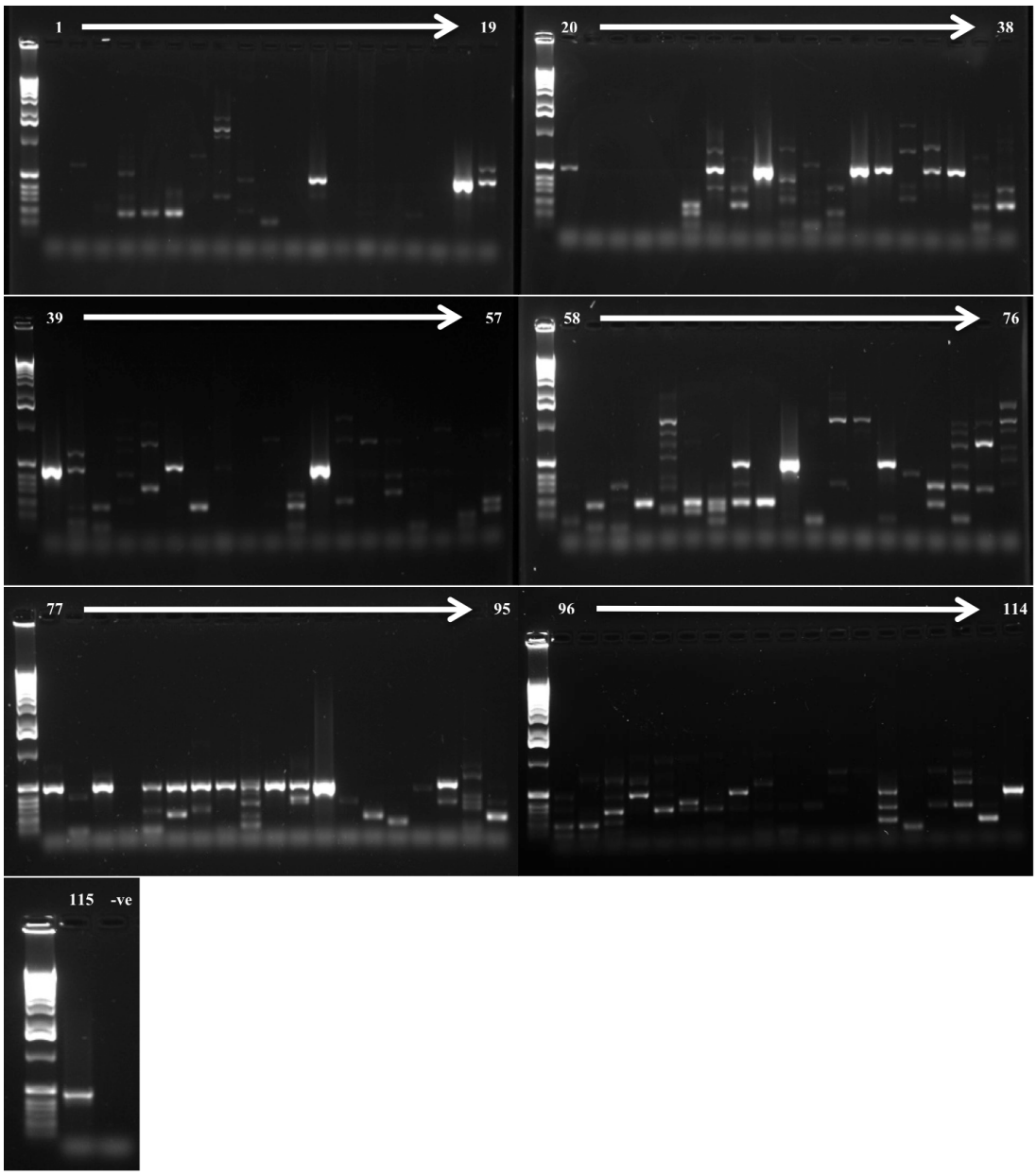


Figure 5.6*a* *AMT1* screening results: PCR product for *AMT1* visualised on agarose gels for 115 *Alternaria alternata* isolates and a negative control. Lane numbers are marked on gels. A single 458 bp amplicon (as present in lane 77) confirmed the presence of *AMT1*.

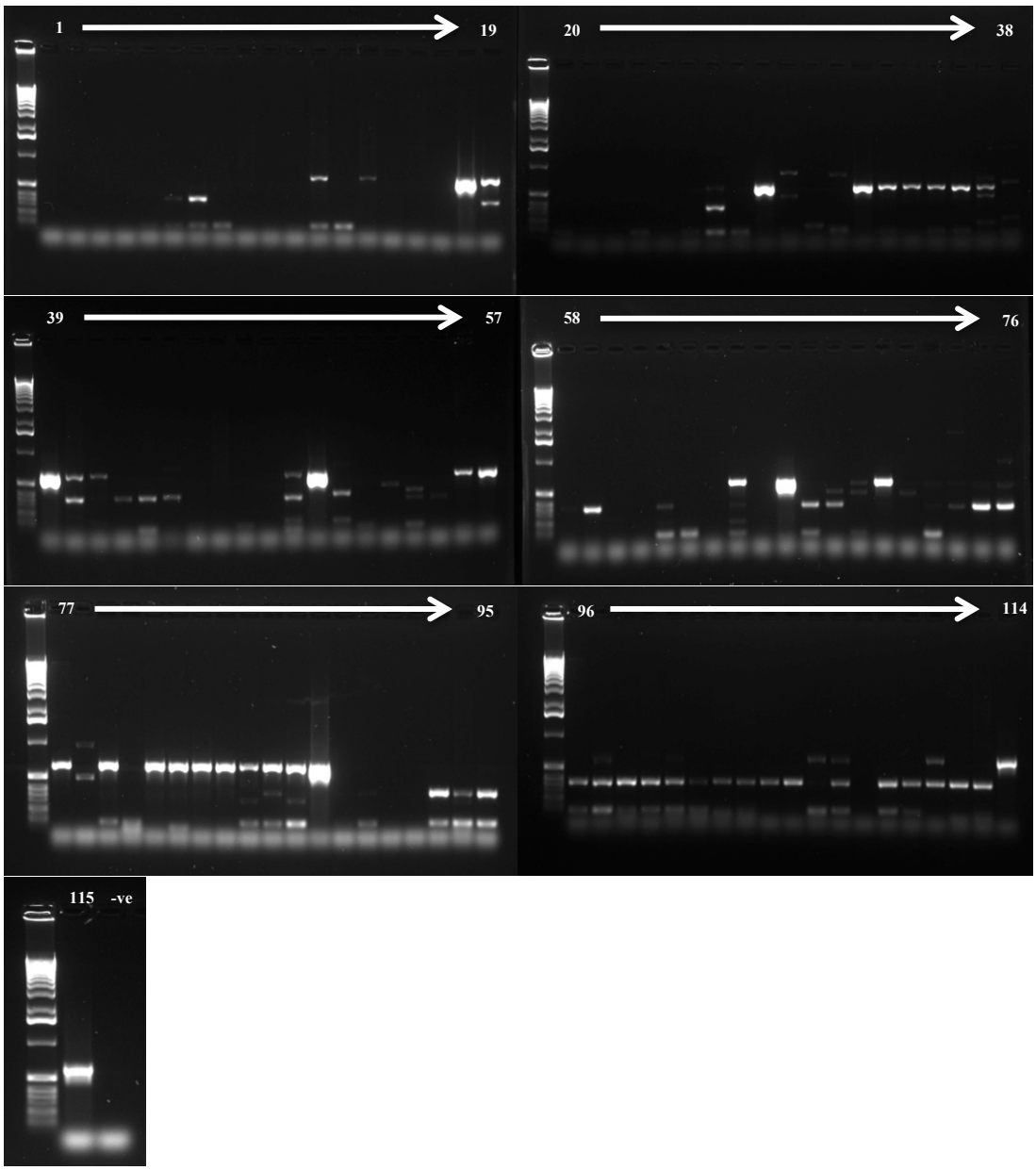


Figure 5.6*b* *AMT2* screening results: PCR product for *AMT2* visualised on agarose gels for 115 *Alternanria alternata* isolates and a negative control. Lane numbers are marked on gels. A single 613 bp amplicon (as present in lane 77) confirmed the presence of *AMT2*.

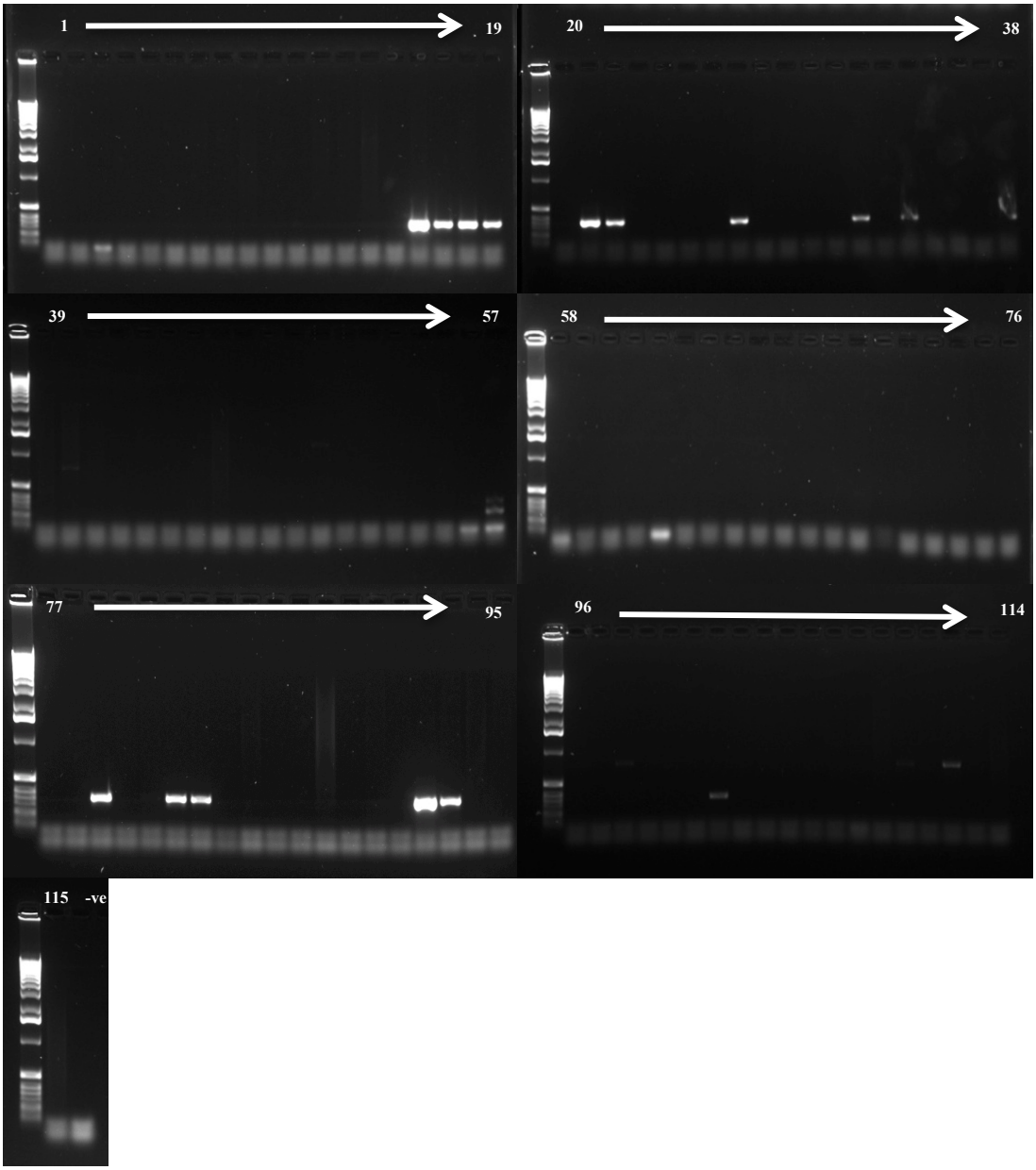


Figure 5.6*c* screening results: PCR product for *AKT1* visualised on agarose gels for 115 *Alternaria alternata* isolates and a negative control. Lane numbers are marked on gels. A single 310 bp amplicon (as present in lane 19) confirmed the presence of *AKT1*.

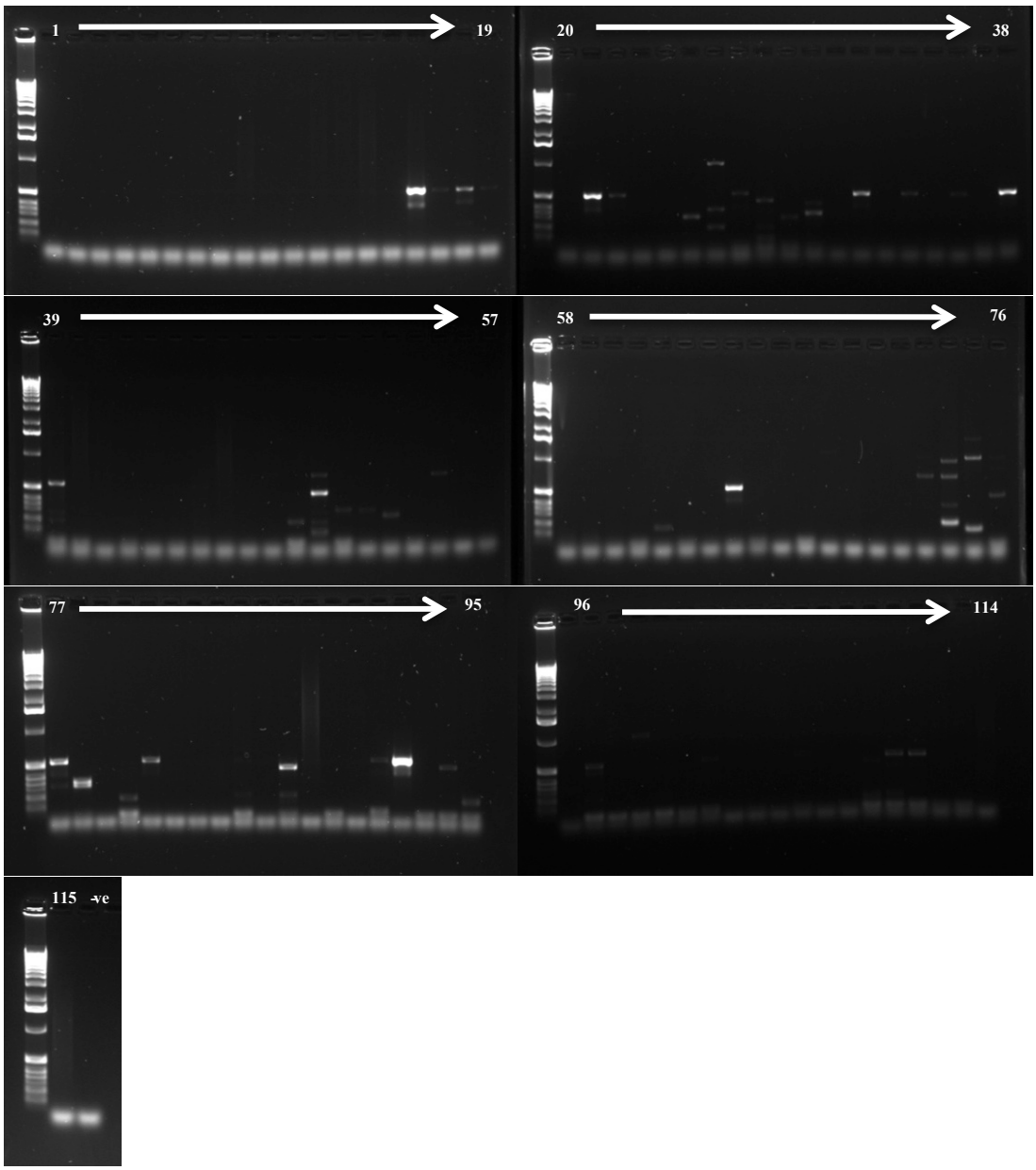


Figure 5.6*d* *AKT2* screening results: PCR product for *AKT2* visualised on agarose gels for 115 *Alternaria alternata* isolates and a negative control. Lane numbers are marked on gels. A single 514 bp amplicon (as present in lane 77) confirmed the presence of *AKT2*.

Distribution of two apple and two pear toxin synthesis genes in a multi-locus phylogeny

Isolates that tested positive for apple toxin genes *AMT1* and *AMT2*, and the pear toxin genes *AKT1* and *AKT2* were distributed throughout the multi-locus phylogeny determined in Chapter 3 (Fig. 5.7).

Clade 1 contained one isolate that tested positive for *AMT1* (*ex.* apple), one isolate that tested positive for *AMT2* and *AKT2* (*ex.* busy lizzy), and four isolates that tested positive for just *AKT2* (two isolates *ex.* apple and two isolates *ex.* pear).

Clade 2 contained one isolate that tested positive for just *AMT1* (*ex.* pear) and five isolates that tested positive for just *AMT2* (three *ex.* apple, one *ex.* strawberry and one *ex.* carnation). One isolate tested positive for just *AKT1* (*ex.* apple), one for just *AKT2* (*ex.* pear) and one for both *AKT1* and *AKT2* (was "*AKT1+AKT2* positive"; *ex.* apple). Clade 2 was the only clade to contain isolates that carried both *AMT1* and *AMT2* genes (were "*AMT1+AMT2* positive"; 14 isolates). Ten of these isolates (three *ex.* apple, three *ex.* pear, one *ex.* potato and three were isolated from unknown material) did not test positive for *AKT* genes, while two isolates were also *AKT1+AKT2* positive (one *ex.* apple and one *ex.* citrus) and two isolates were *AMT1+AMT2* positive and also tested positive for *AKT2* (*ex.* apple). Three isolates tested positive for just the *AMT2* gene and either *AKT1* or *AKT2* genes. Two had *AMT2* and *AKT1* genes (one *ex.* pear and one *ex.* strawberry) and one had *AMT2* and *AKT2* genes (*ex.* watermelon).

Clade *3* comprised three isolates *ex.* Asian pear, each of which were *AKT1+AKT2* positive.

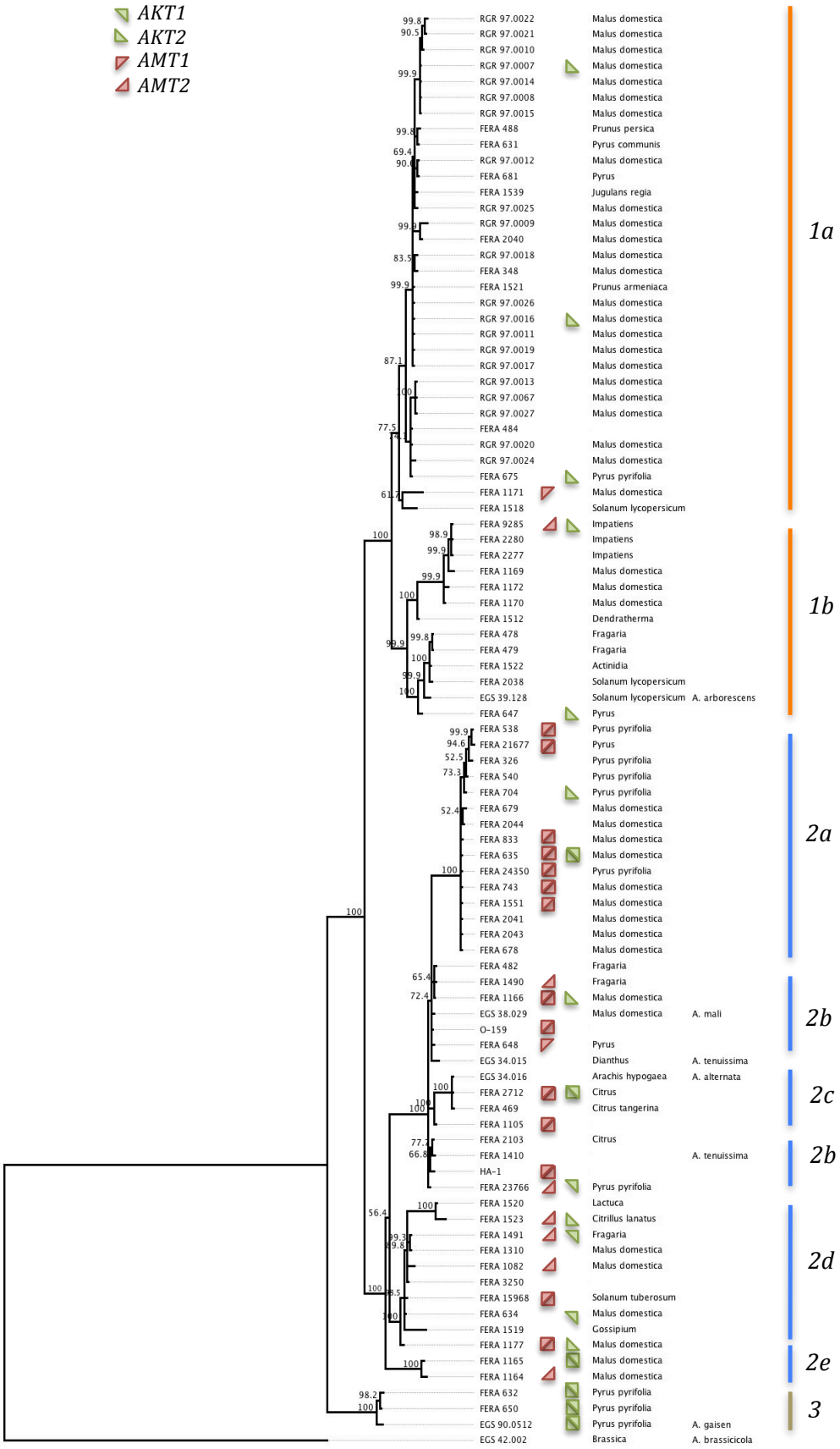


Figure 5.7 Distribution of *AMT* and *AKT* genes throughout a multi-locus phylogeny: Positive detection of PCR product for *AMT1*, *AMT2*, *AKT1* and *AKT2* toxin gene regions for isolates in a phylogeny of the *Alternaria alternata* species group (Fig. 3.2).

BLAST searches of 40 toxin synthesis genes against *Alternaria alternata* genomes

Homology between toxin genes

BLASTn searches of the 40 *A. alternata* toxin sequences against one another identified five sets of highly similar genes with BLAST alignments comprising 96-100% of the original query sequence, of which over 75% of sites were identical and which had e-values less than $1x10^{-1000}$ (Table 5.5). As such, these genes were considered to be homologs of one another (Table 5.5: Homolog groups *a*-*e*). A single apple (*AMT*) gene had a homolog in the strawberry (*AFT*) genes (*AMT2* and *AFTS1*; Table 5.5: group *a*). The four pear toxin (*AKT*) genes had homologs in strawberry (*AFT*) and tangerine (*ACTT*) genes (Table 5.5 groups *b*-*e*): *AKT1*, *AFT1-1* and *ACTT1* (group *b*); *AKT2* and *ACTT2* (group *c*); *AKT3*, *AFT3-1*, *AFT3-2* and *ACTT3* (group *d*); *AKTR*, *AFTR-1*, *AFTR-2* and *ACTTR* (group *e*). No genes were homologs of rough lemon or tomato toxin genes. Apple toxin gene *AMT15* showed a short region of similarity to *AMT14* (38 identical sites in 42 bp: Table 5.5), but this was too short to indicate homology.

Table 5.5 Detection of five homolog groups within 40 *Alternaria alternata* toxin genes: BLASTn searches of 40 toxin genes from six *A. alternata* pathotypes resulted in 16 toxin genes showing similarity to up to three other toxin genes. BLAST alignment length, e-value and number of identical sites are shown for each result.

Blast Queery			Homolog group	Homolog 1				Homolog 2				Homolog 3			
Pathotype	Gene	Length		Homolog	Hit length	E-value	Identical sites	Homolog	Hit length	E-value	Identical sites	Homolog	Hit length	E-value	Identical sites
Apple	*AMT2*	1254	*a*	*AFTS1*	1206	0	924								
	AMT15	555		*AMT14*	42	$7.4x10^{-11}$	38								
Pear	*AKT1*	2028	*b*	*ACTT1*	786	0	707	*AFT1-1*	2028	0	1926				
	AKT2	849	*c*	*ACTT2*	848	0	768								
	AKT3	1061	*d*	*ACTT3*	1061	0	1011	*AFT3-1*	1074	0	1020	*AFT3-2*	1075	0	1020
	AKTR	1335	*e*	*AFTR1*	1335	0	1295	*ACTTR*	1335	0	1262	*AFTR-2*	1335	0	1335
Strawberry	*AFT1-1*	2027	*b*	*AKT1*	2027	0	2027	*ACTT1*	785	0	704				
	AFT3-1	1074	*d*	*AKT3*	1074	0	1020	*ACTT3*	1074	0	998	*AFT3-2*	1075	0	1074
	AFT3-2	1075	*d*	*AFT3-1*	1075	0	1074	*AKT3*	1075	0	1020	*ACCT3*	1075	0	998
	AFTS1	1252	*a*	*AMT2*	1206	0	924								
	AFTR-1	1338	*e*	*ACTTR*	1335	0	1258	*AFTR-2*	1338	0	1338	*AKTR*	1335	0	1295
	AFTR-2	1335	*e*	*ACTTR*	1335	0	1259	*AFTR-1*	1335	0	1334	*AKTR*	1335	0	1296
Tangerine	*ACTT1*	785	*b*	*AFT1-1*	785	0	704	*AKT1*	786	0	707	*AKTR*	1335	0	1295
	ACTT2	849	*c*	*AKT2*	848	0	768								
	ACTT3	1061	*d*	*AFT3-2*	1075	0	998	*AFT3-1*	1074	0	998	*AKT3*	1061	0	1011
	ACTTR	1308	*e*	*AFTR-2*	1335	0	1259	*AKTR*	1335	0	1262	*AFTR-1*	1335	0	1258

Table 5.6*a* Profile of toxin gene BLAST hits against 12 *Alternaria alternata* genomes (including relative copy numbers of hit contigs): Presence of similar regions is marked by the relative copy number of hit contigs to contigs in the N50, those marked in bold were present in over 2.5 times the copy number of N50 contigs. Homologous toxins are marked (*a*-*e*). Genomes are separated by their position in a multi-locus phylogeny (Fig.3.7). Results marked * showed low similarity to the query sequence. Alignments marked n/a were to a misassembled contig, from which coverage could not be determined.

BLAST query			Homolog group	Presence in genome (marked by relative copy number)											
				Clade 1			Clade 2								Clade 3
Pathotype	Gene	Length		*FERA 675* ex. pear	*RGR 97.0013* ex. apple	*RGR 97.0016* ex. apple	*FERA 648* ex. pear	*FERA 1082* ex. apple	*FERA 1164* ex. apple	*FERA 24350* ex. pear	*FERA 635* ex. apple	*FERA 743* ex. apple	*FERA 1166* ex. apple	*FERA 1177* ex. apple	*FERA 650* ex. pear
Apple	*AMT1*	13092									**2.7**	**3.1**	**2.9**	**7.6**	
	AMT2	1254	*a*	*	*	*	*	*	*	*	**3.6**	**4.5**	n/a	**11.3**	*
	AMT3	1561									**3.8**	**4.4**	1.2	**10.2**	
	AMT4	913									**3.8**	**4.1**	n/a	**12.8**	
	AMT5	1223									**3.8**	**4.1**	n/a	**11.3**	
	AMT6	1403									**3.8**	**4.1**	n/a	**12.0**	
	AMT7	1797		*	*	*	*	*	*	*	**3.8**	**4.1**	n/a	**12.0**	*
	AMT8	2829									**3.8**	**4.1**	n/a	**11.6**	
	AMT9	1411									**3.8**	**4.1**	n/a		
	AMT10	2703									1.9	2.0	1.2	**3.4**	
	AMT11	1008		*	*	*	*	*	*	*	1.9	1.6	1.9	**3.7**	*
	AMT12	1248									1.9	1.6	1.9	**3.7**	
	AMT13	497									**2.9**	2.1	1.1	**3.5**	
	AMT14	1105									**2.9**	2.1	1.1	**3.5**	**5.0**
	AMT15	555		**6.7**	1.0		1.0	2.0	2.0	1.0		**4.2**	1.7	**3.0**	1.0
	AMT16	1183		1.0	1.0	1.0	1.0	1.0	1.0	1.0	**2.9**	2.1	1.1	**3.5**	**1.0**
	AMTR1	2461		1.0	1.0	1.0	1.0	1.0	1.0	1.0	**2.9**	2.0	1.2	**3.4**	**5.0**
Pear	*AKT1*	2028	*b*												**7.8**
	AKT2	849	*c*												**9.1**
	AKT3	1061	*d*												**8.4**
	AKTR	1335	*e*												**9.1**

Table 5.6*b* Profile of toxin gene BLAST hits against 12 *Alternaria alternata* genomes (including relative copy numbers of hit contigs): Presence of similar regions is marked by the relative copy number of hit contigs to contigs in the N50, those marked in bold were present in over 2.5 times the copy number of N50 contigs. Homologous toxins are marked (*a*-*e*). Genomes are separated by their position in a multi-locus phylogeny (Fig.3.7). Where BLAST results returned alignments with low similarity to query sequences, presence is marked by an *. Alignments marked n/a were to a misassembled contig, from which coverage could not be determined.

BLAST query			Homolog group	Presence in genome (marked by relative copy number)											
				Clade 1			Clade 2								Clade 3
Pathotype	Gene	Length		*FERA 675* ex. pear	*RGR 97.0013* ex. apple	*RGR 97.0016* ex. apple	*FERA 648* ex. pear	*FERA 1082* ex. apple	*FERA 1164* ex. apple	*FERA 24350* ex. pear	*FERA 635* ex. apple	*FERA 743* ex. apple	*FERA 1166* ex. apple	*FERA 1177* ex. apple	*FERA 650* ex. pear
Strawberry	*AFT1-1*	2027	*b*												**7.8**
	AFT3-1	1074	*d*												**8.4**
	AFT3-2	1075	*d*												**8.4**
	AFT9-1	9073													*
	AFT10-1	1843						*	*		*	*	*	*	*
	AFT11-1	1746													**2.7**
	AFT12-1	894													1.9
	AFTS1	1252	*a*	*	*	*	*	*	*	*	**3.6**	**4.5**	n/a	**11.3**	*
	AFTR-1	1338	*e*												**9.1**
	AFTR-2	1335	*e*												**9.1**
Tangerine	*ACTT1*	785	*b*												**3.6**
	ACTT2	849	*c*												**7.8**
	ACTT3	1061	*d*												**8.4**
	ACTTR	1308	*e*												**9.1**
	ACTT5	1883													*
	ACTT6	897													**3.9**
Rough lemon	*ACRTS1*	1394		1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
	ACRTS2	7958													
Tomato	*ALT1*	668													

Presence of toxin genes in 12 *Alternaria alternata* genomes

The genomes of four strains *ex.* apple (*FERA 635*, *743*, *1166* and *1177*) contained regions with high similarity to 13-15 of the 16 genes involved in apple toxin synthesis, and also to the *AMT2* homolog, *AFTS1* (Table 5.6). These 13-15 *AMT* genes were considered to be present in the four strains due to BLAST alignments comprising over 80% of the original query sequence, containing a high number of identical nucleotides and having e-values less than $1x10^{-100}$ (Appendix, Table 8.1: *a*, *e*, *h*, *i*). An exception to this was the *AMT1* gene alignments, which comprised 35-100% of the original 13,092 bp query sequence. The shortest of these (35%; *FERA 1177*) was to a 4601 bp contig, indicating that the gene was present in the *FERA 1177* genome but had only been partially assembled in the genome assembly (Appendix, Table 8.1: *i*). *AMT3* and *AMT5* alignments to *FERA 1177* were also exceptions with alignments comprising 25 and 19% of query sequences (respectively). Visual examination indicated that these alignments were also made to partially assembled genes. *FERA 635*, *743*, *1166* and *1177* genomes also contained the *AMT2* gene rather than its homolog *AFTS1*, with *AMT2* alignments showing 99-100% identical sites, rather than 77% within *AFTS1* alignments (Appendix, Table 8.1: *a*, *e*, *h*, *i*). As such these strains were considered to contain the apple toxin gene cluster (were "*AMT* positive").

Strain *FERA 650* (*ex.* pear) contained regions with high similarity to the four genes involved in pear toxin synthesis and their homologs in strawberry and tangerine pathotypes (Table 5.6). Pear toxin genes were considered to be present in the genome with alignments containing greater numbers of identical sites (99-100%) than those of strawberry (95-97%) and tangerine homologs (80-95%; Appendix, Table 8.1: *c*). The *FERA 650* genome also contained regions with high similarity to seven genes involved in toxin synthesis for apple (*AMT14*), strawberry (*AFT9-1*, *AFT10-1*, *AFT11-1* and *AFT12-1*) and citrus (*ACTT5* and *ACTT6*) pathotypes (Table 5.5). The alignment of the apple toxin gene *AMT14* comprised 91% of the query sequence and contained 80% identical sites (Appendix, Table 8.1: *c*). The strawberry toxin genes *AFT9-1*, *AFT10-1*, *AFT11-1* and *AFT12-1* had alignments comprising 55, 72, 97 and 100% of the query sequence (respectively) and had 97, 97, 96 and 86% identical sites (Appendix: Table 8.1). The contig that *ACTT9-1* aligned to was shorter than the query

sequence, indicating that this gene was partially assembled (Appendix, Table 8.1: *h*). The citrus toxin genes *ACTT5* and *ACTT6* had alignments comprising 100 and 98% of the query sequence (respectively) and had 91 and 92% identical sites (Appendix: Table 8.1). As such this strain was considered to contain the pear toxin gene cluster (was "*AKT* positive").

Regions showing high similarity to toxin genes in the genomes of *FERA 635*, *743*, *1177* and *650* were often present on contigs that had a mean coverage (during genome assembly) over 2.5 times that of contigs in the N50 (Table 5.6), indicating that these contigs may be present in multiple copies in the genome. Toxin genes often showed similarity to the same contig, for example six toxin genes (*AMT4*, *AMT5*, *AMT6*, *AMT7*, *AMT8* and *AMT9*) all showed high similarity to regions in *FERA 635* contig 2 (13,866 bp).

Contig *599*, of strain *FERA 1166* was observed to be mis-assembled and as a result, coverage data was not collected for toxin genes (Table 5.6). Alignments of seven *AMT* genes (Appendix: Table 8.1, *h*) were present on a 0.9 Mb contig. Visual inspection of the contig found that these alignments were positioned in the first 23 Kb of sequence data. Base pairs 23,119-23,292 were flanked by sequences of unknown bases ("N"s) and corresponded with a drop in coverage of genome sequencing reads from approximately 100 times coverage to 20 times coverage.

All genomes contained regions with sequence similarity to toxin genes *AMT2*, *AMT7*, *AMT11* and *AFTS1*, however strains *FERA 648*, *675*, *1082*, *1164*, *24350* and *RGR 97.0013* and *97.0016* had low similarity alignments comprising 6-25% the query sequence (Appendix, Table 8.1: *b*, *d*, *f*, *g*), and were not considered to indicate presence of these toxin genes (Table 5.6). These regions were not present in high copy number in genomes, as they were within contigs that had a mean coverage (during genome assembly) 1-2.4 times that of contigs in the N50 (Table 5.6).

All genomes contained regions with high similarity to *AMT16* and *AMTR1*. In genomes that did not contain other apple toxin genes, BLAST alignments comprised 96-100% of the query sequences and showed 69-90% sequence identity. The copy number of these genes was indicated to be similar to that of contigs in the N50 for all genomes that did not carry apple toxin genes. All genomes contained a region with high similarity to the rough lemon *ACRTS1* comprising 80% the length of the query

length and contained 65% identical sites. A region showing similarity to AMT15 was present in most strains, with BLAST alignments comprised 46-57% of the query sequences and showed 70-72% sequence identity.

The 310-311 bp region of similarity to the *AMT2* gene, present in genomes that were not *AMT* positive (*FERA 675*, *648*, *1082*, *1164*, *24350* and *RGR 97.0013* and *97.0016*) included a region of 20 bp with similarity to the primer site for AMT-2f2 (Table 2.2). 12 sites were identical to the primer sequence, whereas nucleotides at positions 1-2, 7-10 and 19-20 were different.

The presence of *AMT* and *AKT* toxin genes in the genomes of the 12 strains (Table 5.6) was observed to be different from those results obtained from PCR screens for *AMT1*, *AMT2*, *AKT1* and *AKT2* (Table 5.7).

Table 5.7 Conflict between PCR screens for toxin genes and results from genome sequencing: Detection of two apple (*AMT*) and two pear (*AKT*) toxin synthesis genes from PCR screens (Table 5.4), and detection of toxin gene clusters of BLASTn searches of 16 apple and four pear genes against the genomes of 12 strains (Table 5.6). Strains separated by phylogenetic clade (Fig. 3.2).

Strain			PCR results				Genome searches	
			AMT1	*AMT2*	*AKT1*	*AKT2*	*AMT* genes	*AKT* genes
Clade 1	***FERA 675***	*ex. pear*				√		
	RGR 97.0013	*ex. apple*						
	RGR 97.0016	*ex. apple*				√		
Clade 2	***FERA 648***	*ex. pear*	√					
	FERA 1082	*ex. apple*		√				
	FERA 1164	*ex.apple*		√				
	FERA 24350	*ex. pear*	√	√				
	FERA 635	*ex. apple*	√	√	√	√	√	
	FERA 743	*ex. apple*	√	√			√	
	FERA 1166	*ex. apple*	√	√		√	√	
	FERA 1177	*ex. apple*	√	√		√	√	
Clade 3	***FERA 650***	*ex. pear*			√	√		√

Virulence assay for isolates possessing apple toxin-synthesis genes

Pathogenic *A. alternata* isolates were characterised by the formation of brown lesions formed at 14 dpi (Fig. 5.8). Apart from forming lesions at points of inoculation, leaves maintained a healthy appearance during the period. Apple *cv.* Spartan and *cv.* Bramley's seedling leaves showed similar patterns in disease severity, but *cv.* Bramley's seedling had fewer lesions per leaf than *cv.* Spartan.

In Spartan, *AMT* positive isolates (*FERA 635*, *743*, *1166*) caused more lesions on leaves than isolates that were *AMT* negative (*FERA 648*, 1082, *1164*; a mean of 3.53 lesions per leaf in comparison to 0.1, respectively). SDW treatments caused a mean of 0.07 lesions per leaf. The isolates within treatments showed similar responses apart from isolate *FERA 743* within the *AMT* positive treatment, which produced fewer lesions than *FERA 635* and *FERA 1166* (a mean of 2.5 lesions per leaf in comparison to 4.3 and 3.8 respectively).

In Bramley's seedling, *AMT* positive isolates caused more lesions on leaves than isolates that were *AMT* negative (a mean of 2.53 lesions per leaf in comparison to 0.1 respectively). SDW treatments caused a mean of 0.03 lesions per leaf. The isolates within treatments showed similar responses apart from isolate *FERA 743* within the *AMT* positive treatment, which produced fewer lesions than *FERA 635* and *FERA 1166* (a mean of 1.4 lesions per leaf in comparison to 3.5 and 2.7 respectively).

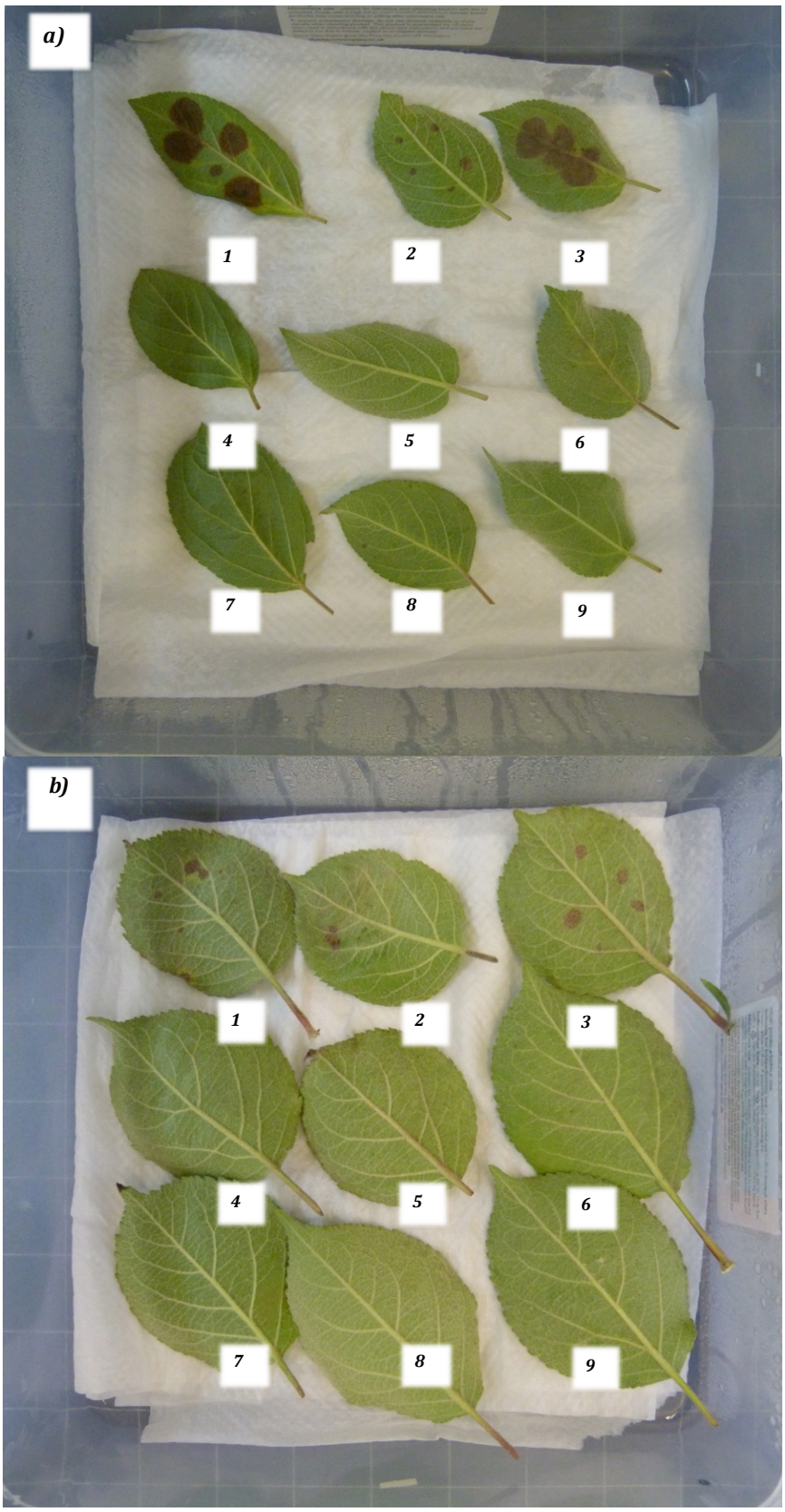


Figure 5.8 Representative images of *a) cv.* Spartan or *b) cv.* Bramley's Seedling apple leaves at 14 dpi. Inoculation of leaves in position: *1-3* with isolates *FERA* 635, *FERA* 743, *FERA* 1166 carrying both AMT toxin genes; *4-6* with isolates *FERA* 648, *FERA* 1082, *FERA* 1164 not carrying AMT toxin genes; *7-9* with water as controls.

Statistical analysis

GLMs for Spartan and Bramley's seedling data showed good fit to the original data. Significant terms identified in the Spartan model were Treatment (F_{72df} =100.64), and Isolate (F_{72df} =2.71) whereas significant terms identified in the Bramley's Seedling model were Treatment (F_{72df} =69.64), Isolate (F_{72df} =3.29), and Box replicate (F_{72df} =2.5). The effects of treatment and isolate were identified through predictive modelling. The effect of replicate box was controlled for, but was not investigated further.

Predictive modelling from the Spartan model (Fig. 5.12: *a*), showed that the *AMT* positive treatment induced significantly more lesions per leaf on average the *AMT* negative treatment and isolates that did not carry both toxin genes (F_{72df} =100.64). Within the *AMT* positive treatment, isolate *FERA 743* produced significantly fewer lesions on average per leaf than isolates *FERA 1166* and *FERA 635* (F_{72df}=2.71).

Predictive modelling from the Bramley's Seedling model (Fig. 5.12: *b*), showed that the *AMT* positive treatment induced significantly more lesions per leaf on average then the *AMT* negative treatment and isolates that did not carry both toxin genes (F_{72df} =69.64). Within the *AMT* positive treatment, isolate *FERA 743* produced significantly fewer lesions on average per leaf than isolates *FERA 1166* and *FERA 635* (F_{72df} =3.29).

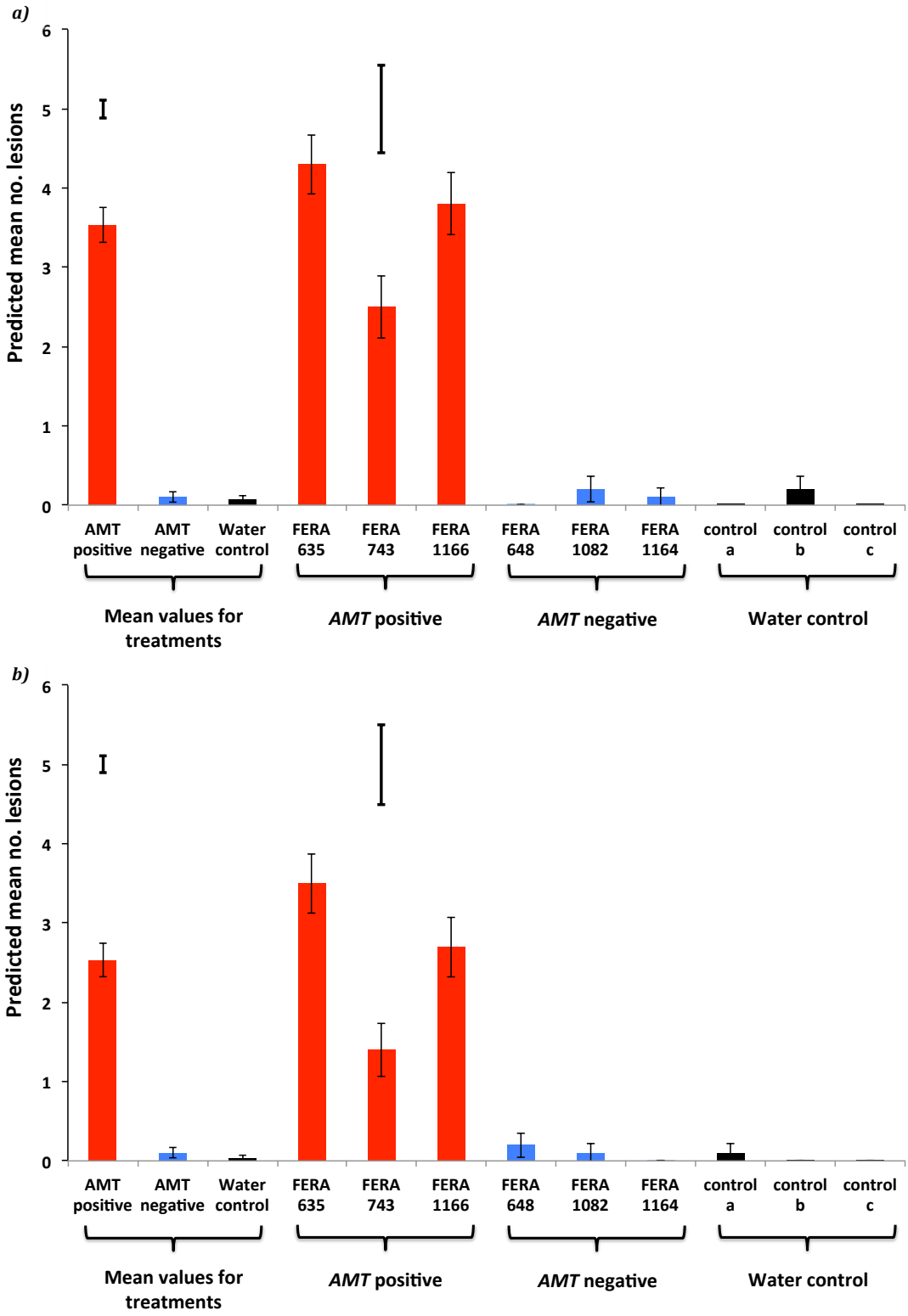


Figure 5.9 Predicted mean number of lesions per leaf for treatments in virulence assays: On *a) cv.* Spartan or *b) cv.* Bramley's seedling leaves infected with *Alternaria alternata* isolates possessing *AMT* toxin genes, not possessing *AMT* toxin genes or water. Mean number of lesions (±SE) as predicted from a GLM and back-transformed at 14 dpi. Predictions are shown for each treatment and isolates nested within each treatment. LSDs are shown in bold bars above significant (P > 0.05) treatments.

5.5 DISCUSSION

Identification of apple and pear pathotypes improves understanding of conditionally dispensable chromosomes

Five homolog groups were identified within the 40 *A. alternata* toxin synthesis genes tested (Table 5.5). This confirmed results of previous studies including Ito *et al.* (2004) who proposed that *AMT* and *AFTS1* are homologs, Masunaka *et al.* (2000) who demonstrated that *AKT1* and *AKT2* are homologs of *ACTT1* and *ACTT2,* and unpublished data (reported by Tsuge *et al.* (2013)) showing that homologs of all currently identified pear toxin genes (including unpublished sequences) are present in strawberry and citrus pathotypes, with the exception of *AKTS1*. Homology was assessed within all forty published HST-synthesis genes in a single experiment, providing the clearest assessment of homology to date. The *AKT* positive isolate *FERA 650* possessed similar regions to *AMT14*, *AFT9*, *AFT10*, *AFT11*, *AFT12* and *ACTT6*, indicating that these genes may have homologs within the pear toxin gene cluster. These may represent unpublished *AKT* genes, mentioned in Tsuge *et al.* (2013) or novel AKT-synthesis genes. The theory that the *A. alternata* CDC was acquired by horizontal gene transfer (HGT) was supported by the recent analysis of codon usage, GC content and predicted gene length in the CDC of the *A. alternata* tomato pathotype (Hu *et al.*, 2012). Similarity between the gene clusters required for pear, citrus and strawberry toxins suggests that the CDCs of these pathotypes may share a closer evolutionary history with each other than they do with the apple pathotype. This may represent multiple, independent horizontal gene transfers occurring into the *A. alternata* species group.

Four *A. alternata* strains were identified as "*AMT* positive" (*FERA 635*, *743*, *1166* and *1177*), possessing 13-15 of the 16 *AMT* genes from genbank accession AB525198.1 (Harimoto *et al.*, 2007). These strains can be considered to represent the *A. alternata* apple pathotype. The strain FERA 650 was "*AKT* positive" possessing the four *AKT* genes currently published (Tanaka *et al.*, 1999, Tanaka and Tsuge, 2000) indicating that this strain represents the *A. alternata* pear pathotype. This is the first study to generate genome sequence data for these two pathotypes. The total lengths of contigs containing *AMT* genes in this study were up to 75 Kb (sum of *AMT*

contig lengths for *FERA 743*: Appendix Table 8.1). This is similar to the *AMT* gene cluster in AB525198.1 that was 117 Kb in length (Harimoto *et al.*, 2007). The genetic content of the remaining 0.93-0.88 Mb apple CDC sequence is unknown. The contigs that *AMT* and *AKT* genes were aligned to were present at up to 12 times the coverage of N50 contigs (Table 5.6), suggesting that toxins are present in multiple copies on the CDC. Gene silencing and knock-outs have suggested that *Alternaria* HST genes are present in three or more copies (Harimoto *et al.*, 2008, Miyamoto *et al.*, 2008). Genetic rearrangement, including gene duplication, has been suggested to play an important role in the evolution of fungal pathogens (de Jonge *et al.*, 2013), and may have played an important role in the evolution of *A. alternata* CDCs. The identification of high numbers of transposons in HST gene clusters for apple and strawberry pathotypes supports this (Hatta *et al.*, 2006, Harimoto *et al.*, 2007). Furthermore, all *Alternaria* possess regions on essential chromosomes with similarity to *AMT16* and *AMTR1*, indicating that there may have been ancestral duplication of these genes onto CDCs and subsequent divergence. If duplication can occur from ECs to CDCs then genetic duplication within CDCs may have occurred. Hu *et al.* (2012) identified 200 genes present in the *A. alternata* tomato pathotype but not in *A. brassicicola*. These were considered to be genes present on the tomato CDC. Further work should aim to replicate this study so that comparisons can be drawn between tomato, pear and apple pathotypes. This will lead to a better understanding of the structure and evolution of these chromosomes.

Phylogenetic distribution of toxin genes

Kusaba and Tsuge (1995b) performed phylogenetics to show that six *Alternaria* pathogens, which had been identified as producing polyketide toxins, had the same ITS haplotype as *A. alternata*. This study is the first time distribution of toxin genes was investigated within an *A. alternata* phylogeny. PCR assays indicated that *A. alternata* isolates carrying *AMT* and *AKT* genes were present in each of the three major clades of the multi-locus phylogeny determined in Chapter 3 (Fig. 5.7). All three isolates present in phylogenetic clade *3* were *AKT1+AKT2* positive, whereas phylogenetic Clade 2 was the only clade to contain isolates that were *AMT1+AMT2*

positive. However conflict was demonstrated between results from PCR screens and genome sequencing (Table 5.7). BLAST searches of toxin genes in 12 *A. alternata* genomes supported the findings that phylogenetic Clade 1 isolates were toxin-gene negative, that Clade 2 contained isolates that were that were *AMT* positive and isolates that were negative and that Clade 3 contained isolates that were *AKT* positive. More confidence was placed in results of genome sequencing data than PCR screens as PCR screens showed multiple banding, possibly leading to false positives.

Phylogenetic Clade 3 contained isolates that were *AKT1+AKT2* positive

The *A. gaisen* morphological reference isolate (*EGS 90.0512*), described in Simmons (2007), has previously been used to represent the *A. alternata* pear pathotype, due to its known production of AKT (Roberts *et al.*, 2011). This isolate was *AKT1+AKT2* positive and AKT positive in genome sequencing. Two other isolates that were also present in phylogenetic Clade 3 were *AKT1+AKT2* positive and *ex.* pear. These isolates did not test positive for either *AMT1* or *AMT2* genes. This supports the naming of this phylogenetic clade *A. alternata* ssp. *gaisen* as this clade shows evidence of representing a lineage of pear specific pathogens.

Phylogenetic Clade 2 contained all *AMT1+AMT2* positive isolates

Isolates that were *AMT1+AMT2* positive were only present in phylogenetic Clade 2. Akamatsu *et al.* (1999) observed that each representative isolate for the seven *A. alternata* pathotypes carried a CDC, less than 1.7 Mb in size, but that non-pathogenic isolates did not have these chromosomes. The theory that complete suites of toxin genes are required for AMT production (and pathogenicity) was supported by this study, with only *AMT* positive isolates (*FERA 635*, *743* and *1166*) causing leaf lesions (Fig. 5.9). Phylogenetic Clade 2 may represent the only lineage containing *A. alternata* apple pathotypes. Only 14 of 42 isolates in this lineage were *AMT1+AMT2* positive. This means that this is not a lineage of exclusively *AMT1+AMT2* positive strains, and therefore molecular markers designed to loci on essential chromosomes

cannot be used to specifically identify the *A. alternata* apple pathotype (isolates possessing the apple CDC).

It is possible that some *Alternaria* isolates may have spontaneously lost apple CDCs. Many cultures in the FERA culture collection were isolated in the early 1990s, and have been stored for 20 years as mycelium inoculated onto agar plugs in water at 4 °C. Repeated sub-culturing (more than ten times) over the course of one year led to the loss of pathogenicity in an apple pathotype isolate (Johnson *et al.*, 2001). The isolate was shown to no longer produce AMT or test positive for *AMT1* and PFGE showed that it no longer carried an additional chromosome (Johnson *et al.*, 2001). A mechanism for loss of CDCs responsible for toxin production has been suggested in *Nectria heamatococca* (Taga *et al.*, 1999). This was based on observations of CDCs in interphase and metaphase *N. heamatococca* cells through fluorescent in situ hybridisation where it was observed that isolates occasionally carried two unattached copies of the CDC in a single nucleus (Taga *et al.*, 1999). It was suggested that this could have been due to nondisjunction of sister chromatids during cell division, leading to one nucleus that carried multiple CDCs and a second nucleus not carrying any CDCs.

Some isolates that were not *ex.* apple also showed the presence of *AMT1* and *AMT2* genes and isolates that were not *ex.* pear showed the presence of *AKT1* and *AKT2* genes. Toxin genes were detected in isolates from busy lizzie (*Impatiens*), strawberry (*Fragaria*), carnation (*Dianthus*), citrus (*Citrus*), watermelon (*Citrullus*) and potato (*Solanum tuberosum*) (Table 5.4). To date, the primers from Johnson *et al.* (2000b) and Roberts *et al.* (2011) have not been widely used to screen isolates outside their target host (apple or pear). In the original paper by Johnson *et al.* (2000b) the *AMT1* primer set was tested against a range of other *A. alternata* pathotypes including pear, strawberry, tomato and tobacco, which all tested negative. The primer set was reported to be able '*to identify the* apple *pathotype from other A. alternata pathotypes, non-pathogenic A. alternata, and other disease causing Alternaria species*'. These primer sets may not be as specific to their target host as originally thought. Alternatively, apple and pear pathotype taxa may have a broader host range than previously thought. The detection of toxin genes in strains not isolated from apple, pear, strawberry or citrus raises questions of whether there are more, unidentified pathotypes in the environment than the seven that are currently recognised (Tsuge *et*

al., 2013). Studies investigating toxin production and presence of toxin genes in strains have typically focussed on individual pathosystems and as such have only screened strains for toxins or toxin genes relating to the pathotype of interest (Johnson *et al.*, 2001, Andersen *et al.*, 2006, Rotondo *et al.*, 2012). Relatively few "reference" strains have been used when studies have screened strains for multiple toxins or toxin genes, such as in Johnson *et al.* (2000b). It is noted that the four isolates confirmed to be *AMT* positive by genome analysis were all *ex.* apple and genome analysis did not support the identification of any isolates in this clade as *AKT1* or *AKT2* positive (Table 5.6).

Phylogenetic Clade 1 only contained isolates testing positive for a single toxin gene

Isolates in phylogenetic Clade 1 only tested positive for single *AMT* or *AKT* toxin genes. Absence of these toxin genes in the genome sequence of two strains indicated that these represented false positives (Table 5.6). Both *AMT1* and *AMT2* genes have been demonstrated to be required for AMT synthesis and similarly *AKT1* and *AKT2* are required for AKT synthesis (Tanaka *et al.*, 1999, Johnson *et al.*, 2000a, Harimoto *et al.*, 2008). Requirement of toxin genes for pathogenicity was confirmed through pathogenicity assays on apple leaves: Isolates that were *AMT* positive (possessing the *AMT* gene cluster) were pathogenic on apple (*cv.* Spartan and *cv.* Bramley's seedling) leaves, whereas isolates that were *AMT* negative were not pathogenic. This may indicate that this is a clade of *A. alternata* strains that do not produce apple or pear HSTs. This may be evidence of niche differentiation between this phylogenetic clade and other phylogenetic clades.

Studies that have sampled *Alternaria* from the field often report many of these strains as non-pathogenic on the host they were sampled from. This was the case in Simmons and Roberts (1993), where it was estimated that only 10% of about 400 isolates isolated from pear orchards in East Asia were pathogenic on a susceptible host. This was also the case in a survey of airborne *Alternaria* spores in Japanese orchards of susceptible pear where 2.5% of 515 isolates produced the pear toxin (AKT) and only one in 575 isolates from a resistant orchard produced AKT (Nishimura *et al.*, 1982). Higher percentages were reported in isolations from apple orchards in Italy but still

only 35% of 44 single spored isolates were able to induce necrosis on unwounded leaves of *cv.* Golden delicious in detached leaf assays (Rotondo *et al.*, 2012).

Isolates within the UoW culture collection, in phylogenetic Clade 1 are recorded as being isolated from apple leaf lesions, but did not test positive for *AMT*-genes. These were isolates with culture IDs beginning in "RGR". These isolations were made from apple *cv.* Pacific rose grown in New Zealand displaying Alternaria blotch symptoms (Fig. 5.10). A similar finding was reported in Rotondo *et al.* (2012) where nine of 44 isolates tested for pathogenicity on apple *cv.* Golden delicious were observed to cause leaf lesions, despite testing negative for the *AMT1* region.

Plant pathogenic interactions are often complex, with suites of effectors and resistance genes typically determining whether a strain of a fungal pathogen will be pathogenic on a particular cultivar of a plant host. Resistance to AMT is reported as being determined by apple cultivars being homozygous or heterozygous for a single dominant allele (Saito and Takeda, 1984). Production of HSTs may be a major factor in pathogenicity in *A. alternata*, but not the only factor.

Figure 5.10 Image of Alternaria blotch on apple *cv.* Pacific Rose caused by *Alternaria* spp. isolates that do not test positive for *AMT* genes: Image was taken by R.G. Roberts (Tree Fruit Research Laboratory, Washington, USA) on plant material in Hastings, New Zealand before performing isolations from these and other representative lesions.

Adachi and Tsuge (1994) suggested that due to *A. alternata* being prevalent in air samples, that saprophytic airborne spores may take advantage of pre-existing necrotic leaf tissue and cause infection. Synergistic effects have been shown between *Alternaria* spp. and other pests. European red mites feed on the upper and lower surfaces of apple leaves, and have been observed in orchards affected by Alternaria leaf blotch that regularly suffer over 60% defoliation through leaf abscission (Filajdic *et al.*, 1995). Filajdic *et al.* (1995) showed that severity of Alternaria leaf blotch was worse in apple orchards in years that European red mites were at high density, and that these two pests had synergistic effects on inducing defoliation in orchards. Interactions between fungal pathogens and other factors such as mechanical damage by insect pests is not necessarily recoded when collecting field specimens. This type of data was not available for this study. It may be the case that mechanical damage or other stresses condition normally non-susceptible hosts to appropriate hosts for non-toxin producing *A. alternata.* In fact, the original report of *A. mali* in the USA in 1924 was of a fungus that was involved in enlarging areas infected by *Botryosphaeria obtusa* or injured by chemicals or other means (Filajdic and Sutton, 1991).

A. alternata have been shown to produce over 30 non-host specific toxins (Robiglio and Lopez, 1995). *A. alternata* apple pathotype isolates have also been shown to be able to produce tentoxin, a nonspecific plant toxin (Scheffer, 1992), in addition to AMT in pure culture (Andersen *et al.*, 2006). All *A. alternata* are considered able to form appressoria to penetrate host cells (Tsuge *et al.*, 2013), and are opportunist necrotrophs as shown by their ability to cause infections in human systems. Tsuge *et al.* (2013) described the function of HSTs in *A. alternata* as being to affect regulation of metabolism, permeability and other key processes, and to suppress the induction of defense responses. '*This potential of HST permits the producer to invade and colonise host cells by conditioning a toxin-sensitive plant as an appropriate host*' (Tsuge *et al.*, 2013). This means that non-HST producing *Alternaria* may be able to act as facultative plant necrotrophs when presented with the right conditions. This questions the suggestion by (Lawrence *et al.*, 2008) that horizontal gene transfer of pathogenicity factors led to the transition of *A. alternata* from a saprophytic to a plant pathogenic lifestyle, but rather allowed transition from opportunist plant pathogen to host-selective plant pathogen.

Origin of conditionally dispensable chromosomes

Three explanations can be considered for the distribution of pathotypes throughout the *Alternaria* phylogeny: (1) *A. alternata* CDCs were present in the last common ancestor of the *A. alternata* species group, before undergoing divergence or loss during vertical transmission; (2) CDCs were acquired in multiple independent events; (3) CDCs originated from the core genome through gene duplication and divergence. Similar explanations were tested for the origin of CDCs ("lineage specific regions") within the *Fusarium oxysporum* f. sp. *lycopersici* genome (Ma *et al.*, 2010). Analysis of genes on lineage specific regions revealed that only 50% of genes had orthologs in essential chromosomes. This along with differences in codon usage indicated that a horizontal gene transfer event had occurred, possibly from related *Fusarium* species (Ma *et al.*, 2010). A difference in codon usage was detected between genes on *A. alternata* tomato pathotype essential chromosomes and the CDC. This study showed that only three of 40 genes involved in HST synthesis had orthologs in *A. alternata* genomes not carrying CDCs. This supports the first two explanations for the origin of CDCs and future work should focus on testing these hypotheses for the origin of *A. alternata* CDCs (Fig. 5.11).

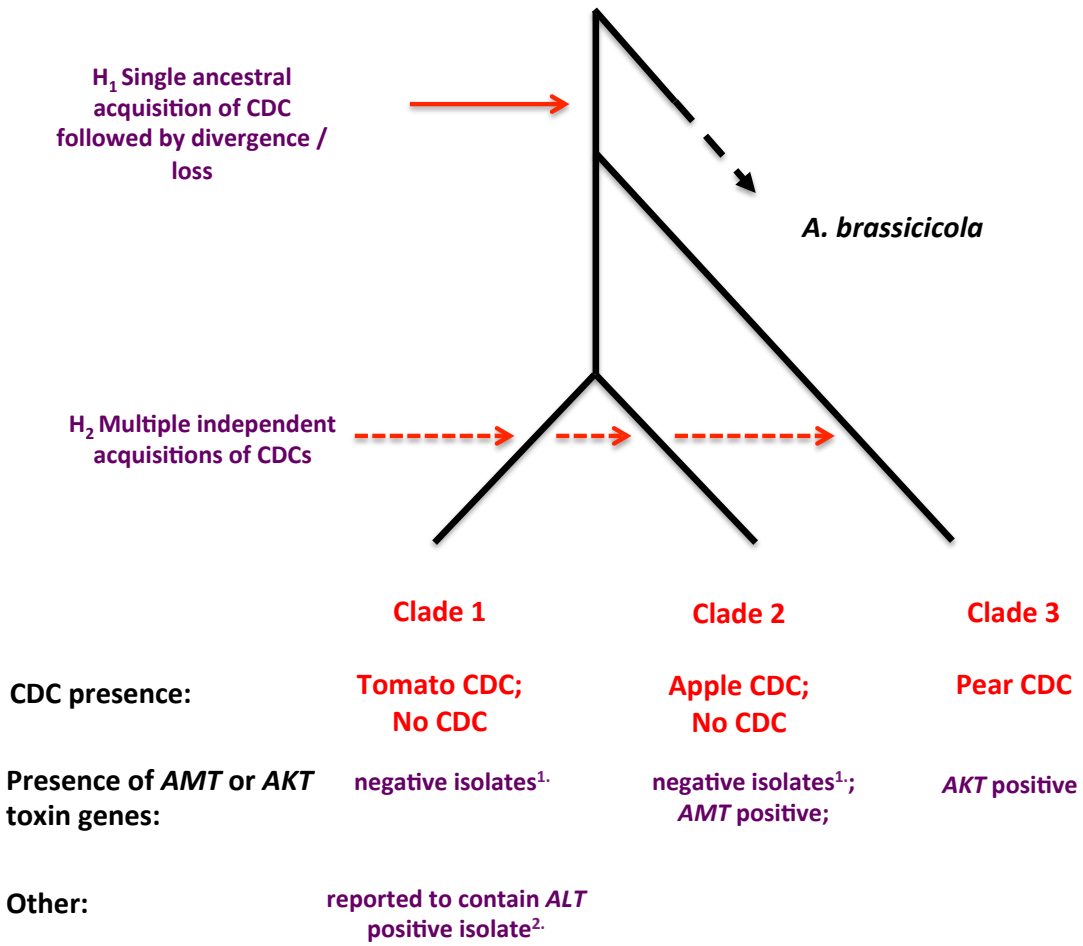


Figure 5.11 Hypotheses for acquisition of conditionally dispensable chromosomes in *Alternaria alternata*: Two hypotheses (H_1 or H_2) for acquisition of CDCs by horizontal gene transfer shown on an idealised phylogeny representing phylogenetic clades identified in Chapter 3 (Fig.3.7). Presence of toxin genes on CDCs of apple (*AMT*) and pear (*AKT*) are shown as determined by genome sequencing and suggested by PCR screens. [1.] PCR assays suggest that *AMT* or *AKT* genes may be present in some isolates. [2.] The *Alternaria arborescens* reference isolate (*EGS 39.128*) carries the tomato pathotype CDC (Hu *et al.*, 2012).

Secondary product in PCR screens

Secondary banding was present in many isolates using all primer sets, particularly *AMT1*. Secondary banding has not been reported in the literature previously. PCR annealing temperature was the same as used in Johnson *et al.* (2000b), annealing temperatures for toxin primers designed in (Roberts *et al.*, 2011) were the same or greater than used in the original paper: *AMT2* (57 °C), *AKT1* and *AKT2* (65 °C). It is possible that other studies have observed secondary banding when using these primers

but did not report it. Andersen *et al.* (2006) used *AMT1* primers by Johnson *et al.* (2000b) in qPCR assays, but confirmed successful amplification of the target region by digestion with restriction enzymes. The same *AMT1* primers have also been used by (Rotondo *et al.*, 2012) who used them for an initial screen for the *AMT1* region. After this initial screen, these researchers developed new primers to flanking regions of the original primers, which were used to screen whether *A. alternata* isolates *ex.* apple had the *AMT1* gene. Although not specifically presenting the distribution of *AMT1* throughout the phylogenies presented in their paper, their data can be used to infer that *AMT1* genes were distributed throughout their phylogenies Rotondo *et al.* (2012). This agrees with PCR results that *AMT1* toxin genes were distributed in multiple phylogenetic lineages (Clades 1 and 2). Primers for the *AMT2*, *AKT1* and *AKT2* gene regions have not been used in any study other than the original work (Roberts *et al.*, 2011). Secondary banding may be due to a number of factors including alternative binding sites in the genome, hybridisation of primers with themselves or the complimentary PCR reaction. BLAST searches of *AMT2* showed that all *AMT* negative isolates contained a potential binding site for the AMT-2f2 primer (Table 5.6). Similarity might be to a secondary metabolism protein on an essential (EC) sharing a functional domain. This could be the case for other primers showing secondary banding. PCR product size was used to determine whether an isolate tested positive for an *A. alternata* toxin gene. Results from PCR screens were valuable in informing the decision of which genomes should be sequenced. These primers are not suitable for identification of *A. alternata* pathotypes in diagnostic laboratories until the basis for this secondary banding is understood.

CHAPTER 6

INVESTIGATING RECOMBINATION IN *ALTERNARIA ALTERNATA*

6.1 INTRODUCTION

Fungal mating systems

Understanding what factors affect an organism's reproductive mode is of fundamental importance because patterns of inheritance affect evolutionary processes (Billiard *et al.*, 2012). Sexual reproduction in fungi requires two individuals possessing opposing "mating types". A single mating type ("*MAT*") genomic region regulates sexual reproduction in Ascomycete fungi consisting of up to two loci referred to as *MAT1-1* and *MAT1-2*. A species may contain individuals carrying either one of these two mating type loci or both. Species that consist of isolates carrying one *MAT* locus are termed heterothallic, while those carrying both mating types are homothallic. The two mating type loci are referred to as idiomorphs, rather than alleles, due to their low genetic similarity and their comprising different genes (Billiard *et al.*, 2012).

When two heterothallic fungi meet that possess opposing mating type idiomorphs then sexual reproduction can occur (Glass *et al.*, 1988, Turgeon, 1998, Berbee *et al.*, 2003). Homothallic individuals, possessing both mating type idiomorphs can mate with another compatible individual or itself. Other mating strategies are also employed in fungi for example, species may be pseudo-homothallic, where they are homothallic but may produce self-sterile heterothallic offspring, which can mate with compatible individuals (Turgeon, 1998). Another strategy has been demonstrated in *Saccharomyces cerevisiae* (Haber, 1998) termed "mating type switching"; this involves a species possessing a single mating type locus but also having copies of both *MAT* idiomorphs elsewhere in the genome, which can be cut and pasted into the *MAT* locus causing the mating type to switch. Occasionally same sex mating, where fungi have initiated mating with another strain of the same mating type, has been reported, as described in *Cryptococcus neoformans* (Lin *et al.*, 2005). Its occurrence in nature has been questioned, but it has been shown as the mechanism by which a novel, highly virulent strain of *Cryptococcus gattii* arose leading to an outbreak of human Meningoencephalitis in 1999, on Vancouver Island Canada (Fraser *et al.*, 2005).

Genes within mating type loci encode transcription factors including high mobility group domain proteins, α-box domain proteins, homeodomain proteins and PPF proteins (Klix *et al.*, 2010, Whittle and Johannesson, 2011). Simple mating type systems such as *Aspergillus fumigatus* (Class Eurotiomycetes) and *Cochliobolus heterostrophus* (Class Dothideomycetes) possess a single mating type gene within each of the *MAT1-1* and the *MAT1-2* loci (Klix *et al.*, 2010, Whittle and Johannesson, 2011). In other species, multiple genes may be present in the mating type locus (Fig. 6.1). Species in the *Alternaria* genus are heterothallic and, similar to *A. fumigatus*, possess single genes in each *MAT* idiomorph (Arie *et al.*, 2000).

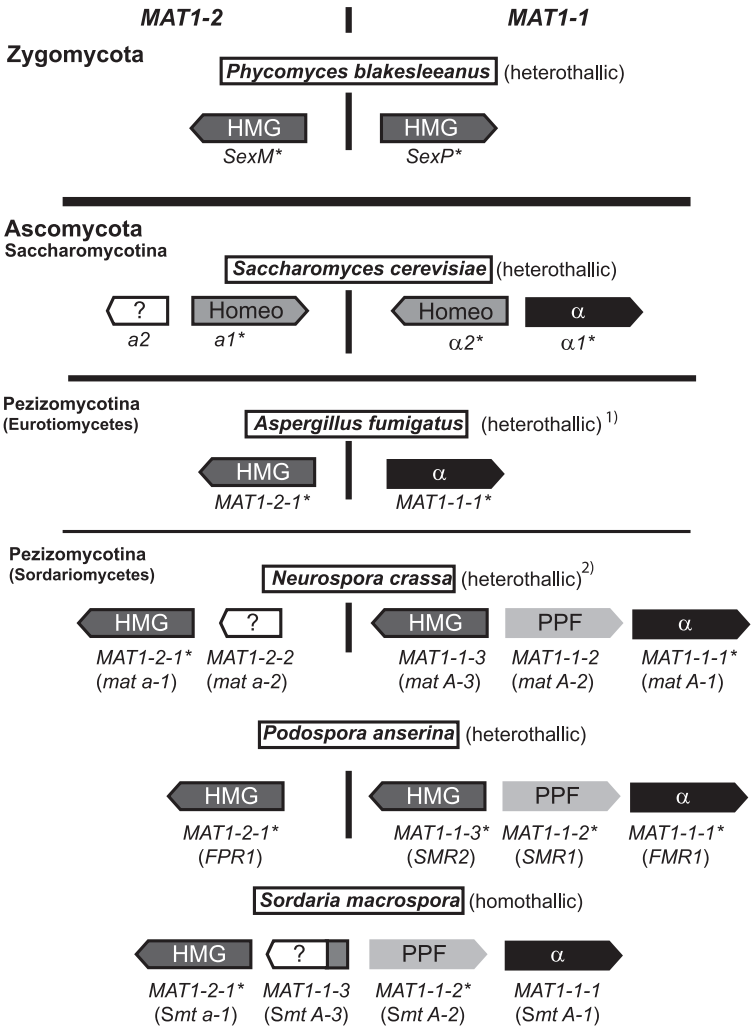


Figure 6.1 Genes present in mating type loci (*MAT1-1* and *MAT1-2*) for Ascomycete and Zygomycete fungi: Arrowed boxes represent orientation and size of genes in the *MAT* locus. Names of each gene and functional domains are presented: HMG domain (HMG); homeodomain (HOMEO); PPF domain (PPF); ? (unknown domain). *Alternaria alternata* posses *MAT1-1-1* and *MAT1-2-1* domains, as presented for *Aspergillus fumigatus*. Image from Klix *et al.* (2010).

Costs and benefits of sexuality

Sexual reproduction can induce fitness costs through unfavourable recombination, costs of finding a mate and energetic transmission when compared to asexual reproduction. Recombination can break locally adapted combinations of alleles at multiple loci, an effect known as the recombination load (Billiard *et al.*, 2012). Homothallic fungi can undergo haploid-selfing where two genetically identical individuals can mate. This system removes the need to find a mate, allowing reproduction with itself or any other individual it encounters in the population, and is considered to have evolved to overcome these costs (Billiard *et al.*, 2012). Even in homothallic fungi where the costs of finding a mate are reduced there are still costs associated with undergoing meiosis and formation of sexual structures, which is more energetically expensive than mitosis (Aanen and Hoekstra, 2007).

Asexual reproduction offers a number of benefits over sexuality in addition to those described above. This includes the ability to produce large numbers of conidia in a shorter amount of time than is required for ascospore production, aiding dispersal of a species (Champe *et al.*, 1994). In addition, asexual spores in *Aspergillus* species are thought to be produced on a wider range of substrates than sexual spores (Dyer and O'Gorman, 2012).

Despite potential fitness costs, sexuality offers a range of benefits over asexuality. The main advantage of sexual reproduction involves the recombination of alleles between genomes which can bring together new favourable and unfavourable combinations of alleles, increasing genetic diversity and allowing selection to occur (Otto, 2009). Sex may also have evolved not only to increase genetic variation but also to purge deleterious mutations. In this case recombination allows the consolidation of alleles that are unfavourable into single genomes that can then be selected against, increasing the efficacy of natural selection (Goddard *et al.*, 2005). The importance of this process has led to it being described as '*the very essence of sex*' (Villeneuve and Hillers, 2001).

Meiotic recombination is a complex process, involving a large number of meiosis-specific and general DNA repair proteins (Villeneuve and Hillers, 2001). The process is not just important in generating genetic diversity within chromosomes, but its

occurrence in meiosis is also essential to ensure chromosomes orientate themselves correctly before chromosomal segregation to occur (Moore and Orr-Weaver, 1998). Meiotic recombination involves a number of steps (Fig. 6.2; presented in Malik *et al.* (2008) and Halary *et al.* (2011)) and can involve up to 86 proteins in fungi (*S. cerevisiae*: Appendix, Table 8.2). Firstly cohesins bind to sister chromatids (Fi. 6.2: A), after which the double-strand breaks are initiated in the DNA (by Spo11 and other enzymes; Fig. 6.2: B) and the beginning of the synaptonemal complex is formed, including Hop1 and later other meiosis-specific proteins. Double strand break repair is initiated (Fig. 6.2: C), and strand exchange proteins are attracted to the double-stand break (Fig. 6.2: D) leading to "strand invasion" of extending strand. This results in a heteroduplex ("holiday junction"; Fig. 6.2: E) that is identified by DNA repair proteins and regulators of crossover frequency. The crossover are resolved either by class II crossovers using meiosis-specific proteins (Mer3, Msh4 and Msh5) or by gene conversion or class I crossovers, which do not (Fig. 6.2: F, G).

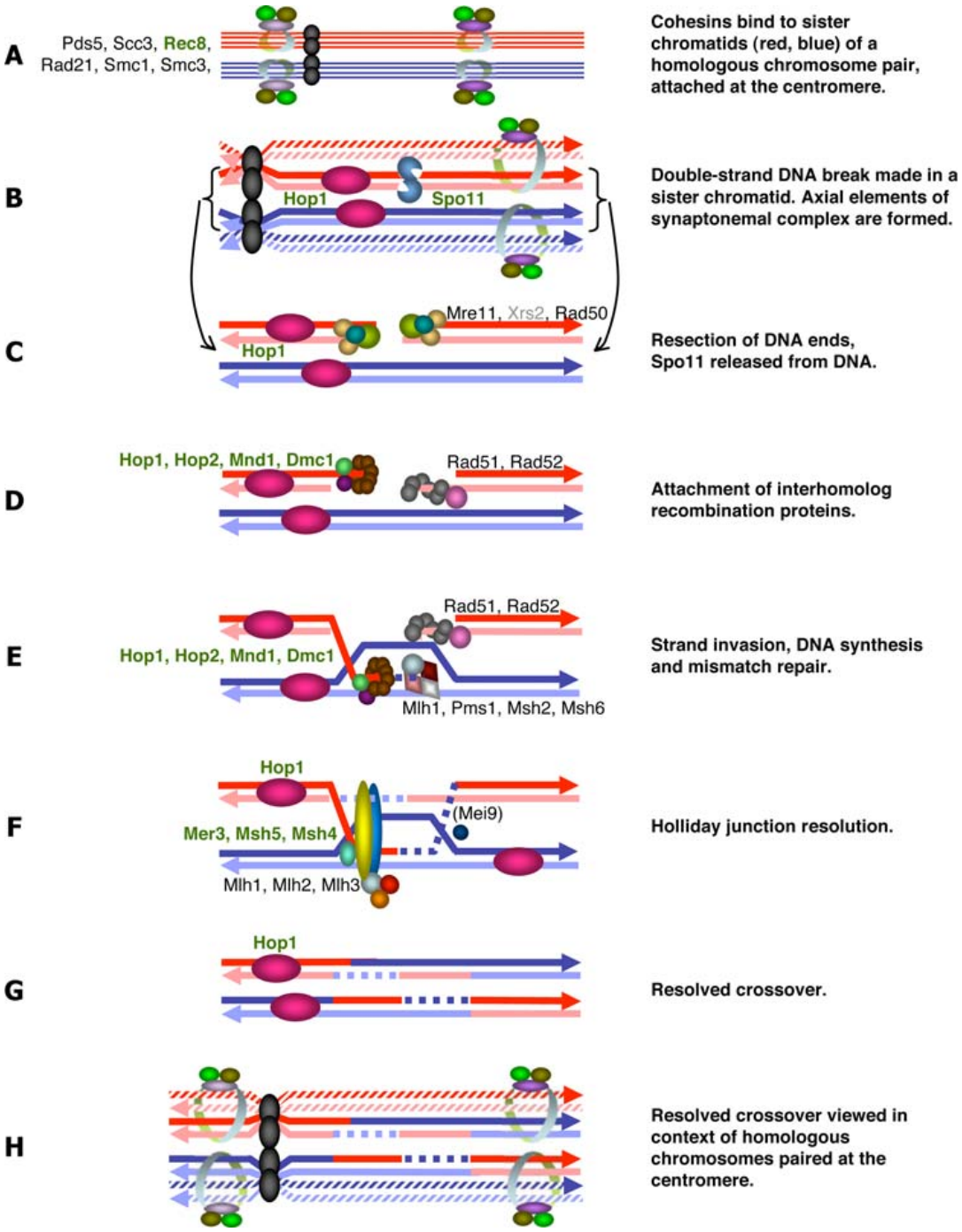


Figure 6.2 Model of meiotic recombination in eukaryotes: As presented in (Malik *et al.*, 2008) detailing the steps of meiotic recombination including cohesin binding (A), generation of double-strand breaks and synaptonemal complex formation (B), initiation of DNA repair (C) and binding of recombination proteins (D), strand invasion and detection (E), its resolution and (F, G) and the resulting recombinant (H). Key meiotic genes are shown including meiosis-specific proteins in green. Functional annotation of these proteins is presented in Appendix, Table 9.2.

Sexual reproduction has shown to have benefits in addition to genetic recombination, which are also specific to fungi. In some ascomycete species, DNA and RNA viruses may be present that are only transmitted via asexual spores. Sexual reproduction may be a means of limiting the presence of these viruses in the genome. Sexual spores of fungi produced through outcrossing or haploid-selfing have been shown to be free of these viruses, as opposed to when these isolates propagate asexually (Coenen *et al.*, 1997, van Diepeningen *et al.*, 2008).

Movement and copying of transposons can have both positive and negative effects on a host genome and organisms employ special mechanisms to control their spread through the genome (Malone and Hannon, 2009). Repeat induced point mutation (RIP) is a process that is unique to fungi that occurs during sex (Galagan and Selker, 2004). It involves single point transitions of G or C nucleotides to A or T nucleotides. These transitions occur in genes that are present in multiple copies, which are repeated in close proximity to one another (repeats). RIP was first shown to occur in *Neurospora crassa* (Selker *et al.*, 1987) and is thought to be a mechanism to inactivate deleterious transposons through non-sense mutations or inducing methylation (leading to down-regulation of a gene) (Selker *et al.*, 1987, Ropars *et al.*, 2012).

Cryptic sexuality

A commonly used statistic is that approximately 20% of fungi are thought to be asexual, based upon the lack of observations of teleomorphic sexual structures (Reynolds, 1993, Taylor *et al.*, 2000). Schurko *et al.* (2009) summarised the problem of identifying species as asexual on this basis: '*how confident can we be that the absence of signs of sex is evidence for no sex at all? It only takes a tiny bit of sex to realise many of its benefits, and rare sex can be difficult to distinguish from the complete absence of sex'*. Cryptic sexuality describes the process(es) where species mate at a low occurrence or in environments that would not normally allow detection.

Sexuality is being identified in many fungi that were previously considered asexual. The clearest sign of mating is observing the formation of a teleomorphic (diploid) life stage from two anamorphic (haploid) fungi. In the absence of such direct observation

then evidence of sexuality may be inferred indirectly. This may involve the identification of functional mating type genes, observation of mating type genes that are maintained in fungal populations in ratios that indicate functionality or finding evidence of recombination within genome sequences. A broad review of identifying sexuality in all organisms by Schurko *et al.* (2009) described how multiple signs of sex can be observed at an organismal-level and at a molecular level. When indirect evidence of sexuality is used, then multiple indicators are required before there is strong evidence of sexuality in an organism (Schurko *et al.*, 2009).

Mating type genes have been shown to be present in supposedly asexual Ascomycetes such as *Fusarium oxysporum, A. fumigatus* and *A. alternata* (Arie *et al.*, 2000, Paoletti *et al.*, 2005). These genes may be present in the genome because a) they are non-functional remnants from sexual ancestors b) they are associated with non-sexual functions c) these fungi are still sexual (Whittle and Johannesson, 2011).

Sexuality has been implied through identification of multiple mating type idiomorphs within a fungal population. Following the loss of sexuality in a species, genetic drift is expected to lead to extinction of a single mating type in fungal populations (Ropars *et al.*, 2012). The fixation of a single genetic polymorphism (such as a mating type idiomorph) in a population is termed lineage sorting (Fig. 6.3).

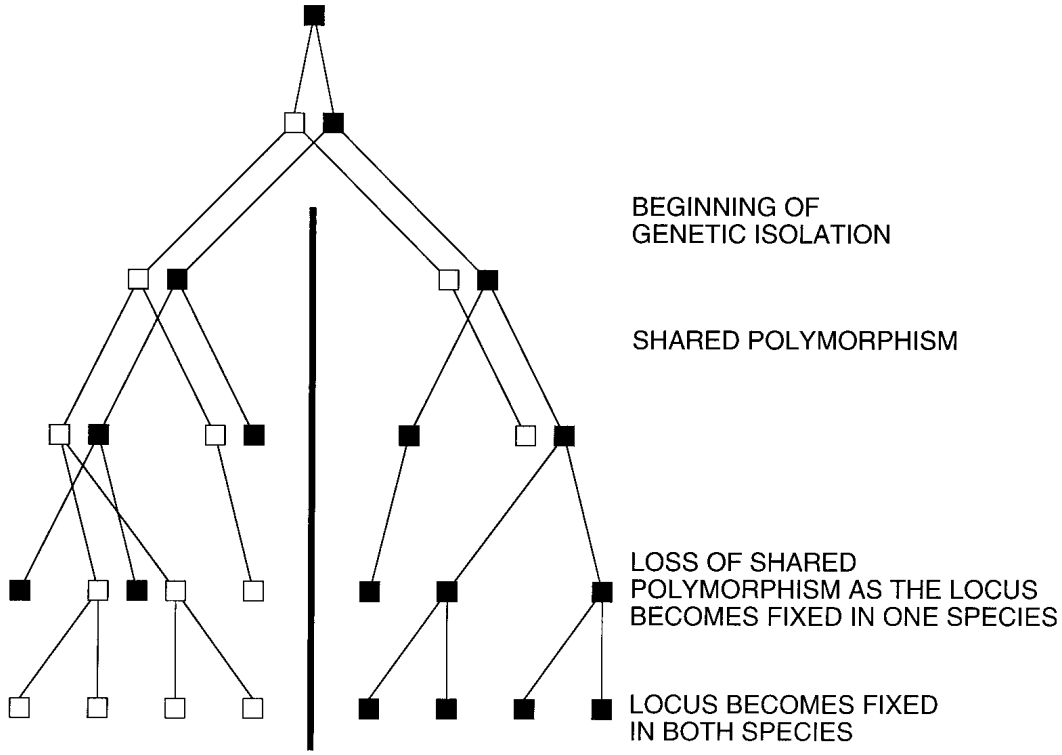


Figure 6.3 Lineage sorting at one polymorphic genetic locus following genetic isolation of two populations: Polymorphism is shared between the two newly isolated species; then it is lost in one species and finally in the other. Image taken from (Taylor *et al.*, 2000).

In a heterothallic sexual population there is a need for two sexually compatible individuals to meet. Frequency dependent selection may act on these populations to maximise the chance of individuals of the opposite mating type meeting (May *et al.*, 1999). This balancing selection can maintain two mating type idiomorphs in equal proportions at low levels of recombination (genetic exchange) (May *et al.*, 1999). An observation of a 1:1 ratio of mating type loci is considered to be strong evidence that sexual reproduction is still occurring in fungal populations (Dyer and O'Gorman, 2012). *Rhynchosporium secalis* is a fungal pathogen of barley, whose teleomorphic state has not been observed and has therefore been considered asexual. Evidence of sexuality was provided by Linde *et al.* (2003) through screening a global population of 1102 isolates. Mating type idiomorphs were found to be present in equal frequencies in populations suggesting frequency dependent selection, which is consistent with sexual reproduction (May *et al.*, 1999, Linde *et al.*, 2003). Frequency dependent selection has also supported evidence for sexuality in other putative asexual opportunistic fungal pathogens including *Aspergillus fumigatus* (Paoletti *et al.*, 2005) and *Mycosphaerella graminicola* (Zhan *et al.*, 2002).

Genome sequencing and functional analysis of genes in model organisms has allowed identification of "core meiotic genes" that are required for meiosis in eukaryotes (Villeneuve and Hillers, 2001). This was initially a list of six meiotic genes but has been subsequently expanded to comprise of 29 genes (Villeneuve and Hillers, 2001, Ramesh *et al.*, 2005, Malik *et al.*, 2008). Within this list are genes for proteins that only have meiotic functions. It is expected that orthologs of meiosis-specific genes would be under relaxed functional constraint in asexual organisms. This would lead to an accumulation of deleterious mutations, such as frameshifts, resulting in them becoming pseudogenes and therefore no longer detectable in the predicted-proteomes of asexual species (Schurko and Logsdon, 2008, Schurko *et al.*, 2009). As such, the presence of meiotic homologues in the genomes of asexual species has been used to indicate capability for sexual reproduction in a species (Ramesh *et al.*, 2005, Malik *et al.*, 2008, Halary *et al.*, 2011, Ropars *et al.*, 2012).

Core meiotic genes have been used to identify homologs in the genomes of protists in the genus *Giardia* (Ramesh *et al.*, 2005) and *Trichomonas vaginalis* (Malik *et al.*, 2008), indicating that these putatively asexual species are capable of meiosis and, thus sexual reproduction (Ramesh *et al.*, 2005, Malik *et al.*, 2008). In a study by Halary *et al.* (2011), the 29 core meiotic genes were expanded to a total of 86 genes that represented all the genes that have meiotic functions in *Saccharomyces cerevisiae*. Using this list Halary *et al.* (2011) searched for meiotic homologs in the genomes of *Glomus* spp. arbuscular mycorrhizal fungi. Detection of homologs to many of the core meiotic genes, including meiosis-specific genes, indicated that these putatively anciently asexual fungi may be capable of a cryptic sexual cycle (Halary *et al.*, 2011). With a growing number of publicly available fungal genomes (Ohm *et al.*, 2012), similar analysis can be performed to investigate whether loss of meiotic genes has occurred in the 20% of fungi that are considered asexual (Reynolds, 1993), or whether they also possess the capability for sexual reproduction.

The functionality of genes involved in sexuality has been used as evidence of sexuality within fungi. Transcriptome sequencing of *Aspergillus fumigatus* showed that pheromone-precursor genes and pheromone-receptor genes were expressed during mycelial growth (Poggeler, 2002). These genes are typically expressed during fungal mating (Poggeler, 2002), and have been considered as evidence for current sexuality in this fungus (Poggeler, 2002, Paoletti *et al.*, 2005).

Genome sequence data can also be investigated for the presence of repeat induced point mutations (RIPs). RIP has formed part of a body of evidence used to infer sexuality indirectly in *Penicillium roqueforti* (Ropars *et al.*, 2012). Ropars *et al.* (2012) used a range of indicators to propose sexuality in a sample of 126 isolates from 38 cheeses from 14 countries: Both mating types idiomorphs were present in fungal populations; *MAT* genes were demonstrated to be under strong purifying selection; whole genome sequencing showed that genes involved in meiosis were conserved within genomes; as well as the presence of repeat induced point mutations.

Parasexual recombination

An alternative method of genetic recombination in fungi is the parasexual cycle. This process involves the formation of a heterokaryon; which is the fusion of nuclei from two haploid individuals into a single diploid nucleus. The diploid nucleus returns to a haploid state by repeated non-disjunction (abnormal segregation) of chromosomes. Recombination occurs by independent assortment of chromosomes and by mitotic crossing over resulting in non-clonal progeny (Milgroom *et al.*, 2009). Parasexuality was first described in *Aspergillus* (*A. niger*) (Pontecorvo *et al.*, 1953), and identified in *Penicillium* (*P. chrysogenum*) shortly after (Pontecorvo and Sermonti, 1954). Parasexuality can be readily induced in the lab for *A. niger* and *Aspergillus nidulans* (Clutterbuck, 1996).

The extent to which parasexual recombination occurs in nature is unclear. Most fungi are able to undergo hyphal fusion with an individual of the same genotype ("self"). However mechanisms are present to prevent heterokaryon formation with "non-self" individuals, known as heterokaryon (or vegetative) incompatibility (Glass *et al.*, 2000). Heterokaryon (in)compatibility is determined by *het* loci and incompatibility at these loci may prevent heterokaryon formation or lead to programmed cell death (Glass *et al.*, 2000, Glass and Dementhon, 2006).

Despite these barriers, there is evidence that parasexuality can occur between genetically different ("non-self") individuals. Parasexuality has been induced in the lab between two *Magnaporthe oryzae* strains with different genotypes (Noguchi *et al.*,

2006) and between two *Fusarium oxysporum* individuals that are heterokaryon incompatible (Molnar *et al.*, 1990). Investigations into the Chestnut blight fungus (*Chryphonectria parasitica*) have shown evidence of heterokaryon formation in nature and that recombination occurred in absence of meiosis (McGuire *et al.*, 2004, McGuire *et al.*, 2005, Milgroom *et al.*, 2009)

Horizontal gene transfer (HGT) is common in prokaryotes and its occurrence is increasingly being recognised in fungi. Strong evidence for HGT was provided through identifying that development of plant pathogenicity in Oomycetes was a result of a HGT from fungi (Richards *et al.*, 2011). Genome sequencing of plant pathogens has provided evidence for HGT within the Dothideomycetes. The toxin gene (ToxA) was part of an 11 Kb region transferred from *Stagonospora nodorum* to *Pyrenophora tricti-repentis*, that allowed host range expansion and the emergence of a new disease of wheat in the 20th Century (Friesen *et al.*, 2006).

HGT has been suggested between the *A. alternata* as a mechanism of genetic exchange within the *A. alternata* (Tanaka *et al.*, 1999, Masunaka *et al.*, 2005, Akagi *et al.*, 2009). Genetic exchange during hyphal anastomosis, a form of parasexual recombination (Clutterbuck, 1996), has been suggested as a mechanism for the formation of an isolate that carries both rough lemon and tangerine pathotype CDCs (Masunaka *et al.*, 2005).

Evidence for sexuality in *Alternaria* spp.

The *Alternaria* genus is ancestrally sexual, with the teleomorphic name for the genus being *Lewia* spp.. *Lewia infectoria*, an early divergent species of *Alternaria* has been shown to produce asci on pseudothecia in the field providing evidence of a teleomorphic stage in the life cycle of *Alternaria* (Perello and Sisterna, 2008). Teleomorphs have been identified in culture for early diverging lineages of the *Alternaria* genus including *Lewia phostica* (Simmons, 2002), *Lewia avenicola* (Kwasna and Kosiak, 2003) and *Lewia hordeicola* (Kwasna *et al.*, 2006).

Recent work has suggested that cryptic sexuality may occur within the *A. alternata* species group. This was suggested following the discovery of mating type loci within

the *A. alternata* and the confirmation that these loci are functional (Arie *et al.*, 2000). *A. alternata* individuals (exhibiting an *A. tenuissima* morphology) have been shown to possess both *MAT1-1* and *MAT1-2* loci, as have *Alternaria brassicicola* and *Alternaria brassicae* (Berbee *et al.* (2003); Fig. 6.4). Recent work by Stewart *et al.* (2011) and Stewart *et al.* (2013) has investigated mating type genes present in North American *A. alternata* populations in the citrus pathosystem. These studies have suggested that *A. alternata* may be undergoing cryptic sexuality or have a recent sexual past (Stewart *et al.*, 2011, Stewart *et al.*, 2013).

Mating type genes have been linked to secondary functions other than mating (Clarke *et al.*, 2001). This includes certain mating types being associated with increased virulence (Lockhart *et al.*, 2005, Zhan *et al.*, 2007). Following the identification of purifying selection acting on mating type genes, Stewart *et al.* (2011) could not rule out mating type genes being maintained in *A. alternata* due to them providing non-sexual benefits.

Dothideomycete genera have been recommended as model organisms for the study of mating type evolution in pathogenic fungi (Turgeon, 1998). Key features of these fungi are their large numbers of species and their diversity of hosts. The distribution of mating type loci within the *A. alternata* species group has not been extensively investigated and is not clearly understood. Recombination could be a mechanism allowing the evolution of pathogens with altered host ranges and hence may be of importance within the *A. alternata*.

Figure 6.4 ITS phylogeny showing sexual ancestry of *Alternaria* spp. and detection of mating type loci in lineages presumed to be asexual: ITS phylogeny of the *Lewia* (teleomorph)/*Alternaria* (anamorph) genus. Image taken from Berbee *et al.* (2003).

6.2. AIMS

This chapter investigates the evidence for recombination within the *A. alternata* species group through the presence and distribution of mating type idiomorphs. Mating type genes *MAT1-1-1* and *MAT1-2-1* genes were used as markers for *MAT*-locus idiomorphs throughout the multi-locus phylogeny determined in Chapter 3. Furthermore the genomes of *Alternaria* spp. were analysed to identify whether they possessed genes required for meiotic recombination.

Specific objectives were to:

1. Use PCR to screen *A. alternata* species group isolates for the presence of *MAT1-1-1* or *MAT1-2-1* genes and investigate their distribution through a multi-locus phylogeny.
2. Test whether mating type ratios deviated from a 1:1 ratio within phylogenetic clades.
3. Use BLAST searching to identify homologs of meiotic genes, as used in Halary *et al.* (2011), in the genomes putatively asexual *A. alternata*, *A. brassicicola* and known sexual Dothideomycetes.

6.3 MATERIALS AND METHODS

PCR screen for mating type genes

The mating type locus of 90 *A. alternata* was detected using gene specific PCR primers. The primer pair AAM1-2 and AAM1-3 was used to amplify a 271 bp fragment of the *MAT1-1-1* gene and the primer pair M2F and M2R was used to amplify a 576 bp fragment of the *MAT1-2-1* gene (Arie *et al.*, 2000). PCR primers were run in multiplex using reagents and reaction conditions as described in Chapter 2 (Table 2.2).

The sizes of PCR amplicons were determined by performing gel electrophoresis and observing DNA migration through a 1% agarose gel. The presence of a single band at 271bp or 576bp confirmed the amplification of *MAT1-1-1* or *MAT1-2-1* loci, respectively. The presence of the *MAT1-1-1* or *MAT1-2-1* genes in an isolate was plotted on the multi-locus concatenated phylogeny as constructed in Chapter 3 (Figure 3.7). Clades were tested to establish whether isolates deviated from a 1:1 ratio of mating types, as assumed for randomly mating populations. The null hypothesis "*Alternaria* isolates within this clade do not significantly deviate from a 1:1 ratio of mating type" was tested within phylogenetic Clade 1 and phylogenetic Clade 2 using a chi-squared test, implemented in Genstat (GenStat, 2011). The test was also performed on the entire *A. alternata* dataset. Phylogenetic Clade 3 contained less than five isolates hence a Chi Squared test was not performed on this clade as there were too few observations for the test to be valid. Statistical tests were performed using a minimum likelihood approach in the statistical software Genstat (GenStat, 2011).

BLAST searches of Dothideomycete genomes for genes involved in meiotic recombination

The predicted proteome for *Saccharomyces cerevisiae* strain *S288C* was downloaded from the Saccharomyces Genome Database (Cherry *et al.*, 2012) and from this, 86 genes with a known meiotic function, as identified in the supplementary material of Halary *et al.* (2011), were extracted. This list included 29 genes identified as core meiotic genes within the eukaryotes (Malik *et al.*, 2008), and fifteen genes that have functional descriptions as meiosis-specific proteins on the Saccharomyces Genome Database (Cherry *et al.*, 2012). The predicted proteome was downloaded for the putatively asexual species *A. brassicicola* (*Abr1*; hosted by the JGI (Grigoriev *et al.*, 2012)) and for three other sexual Dothideomycetes *Pyrenophora tritici-repentis* (*Pt-1C-BFP*: hosted by the Broad institute (Broad, 2013)), *Cochliobolus heterostrophus* (C5: hosted by the JGI (Grigoriev *et al.*, 2012)) *Phaeosphaeria nodorum* (*sn15*: hosted by the JGI (Grigoriev *et al.*, 2012)). Downloaded proteomes along with those predicted for three *A. alternata* strains generated as part of this study (*FERA 650*, *FERA 675* and *FERA 1166*; Chapter 2) were imported into the program Geneious (Kearse *et al.*, 2012), where a BLAST database was made for each proteome. Reciprocal BLASTp searches were performed in Geneious (Kearse *et al.*, 2012) returning the hit with the highest e-value, and with a minimum e-value of $1x10^{-1}$; initially of the 86 *S. cerevisiae* meiotic genes against the target proteome and then of the best hit back against the complete *S. cerevisiae* proteome. Genes from two genomes were considered homologous when they showed greater homology to each other than any other sequence in the opposing proteome.

6.4 RESULTS

Presence and distribution of mating type idiomorphs

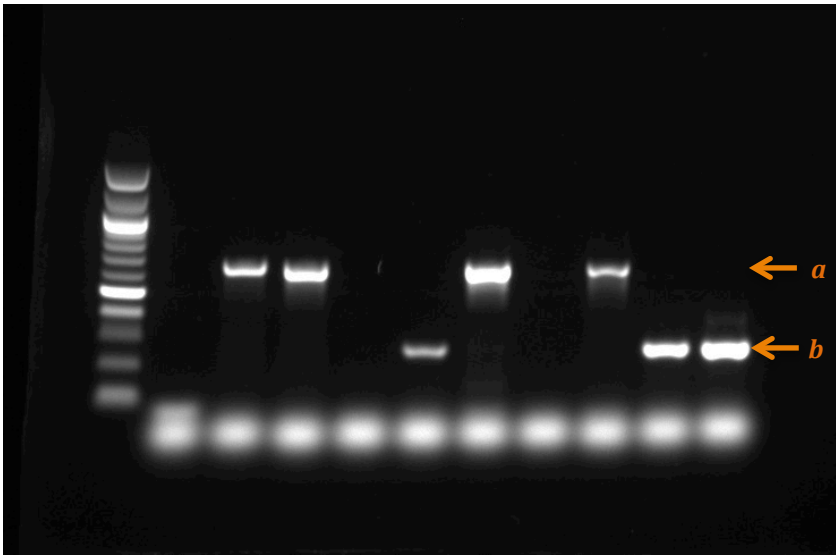


Figure 6.5 Example of mating type PCR results: 576 bp amplicon (*a*) confirmed the presence of the *MAT1-1-1* locus and 271bp amplicon (*b*) confirmed the presence of the *MAT1-2-1* locus. Isolates were heterothallic testing positive one or the other *MAT* idiomorphs. 100bp ladder (New England Biolabs) is shown.

All *A. alternata* species group isolates tested for the presence of *MAT1-1-1* or *MAT1-2-1* were observed to carry a single mating type locus, confirming heterothallism in the species group, possessing one or other of these loci (Fig. 6.5). 42 isolates tested positive for the *MAT1-1-1* locus while 48 isolates tested positive for *MAT1-2-1*. This was not significantly different from a 1:1 ratio of mating type genes, assumed under a random mating population ($\chi^2 = 0.4$; 1df; $P>0.05$).

Distribution of mating type idiomorphs though the major clades in the *A. alternata* phylogeny supported a 1:1 ratio (Fig. 6.6). The null hypothesis that "*Alternaria* isolates within this clade do not significantly deviate from a 1:1 ratio of mating type" could not be rejected within phylogenetic Clade 1 ($\chi^2 = 0.29$; 1df; $P>0.05$) or Clade 2 ($\chi^2 = 0.86$; 1df; $P>0.05$).

Distribution of mating types was observed but not tested within minor clades, as there were too few observations for tests to be reliable (less than five in some clades). Both

mating type genes were observed in six of the eight minor clades encompassing 85 of the 90 observations. The two exceptions were Clade 2*e,* comprising two isolates which both carried the *MAT1-2* idiomorph and Clade 3, comprising three isolates that carried the *MAT1-1-1* idiomorph and including the A. gaisen reference isolate (*EGS 90.0512*).

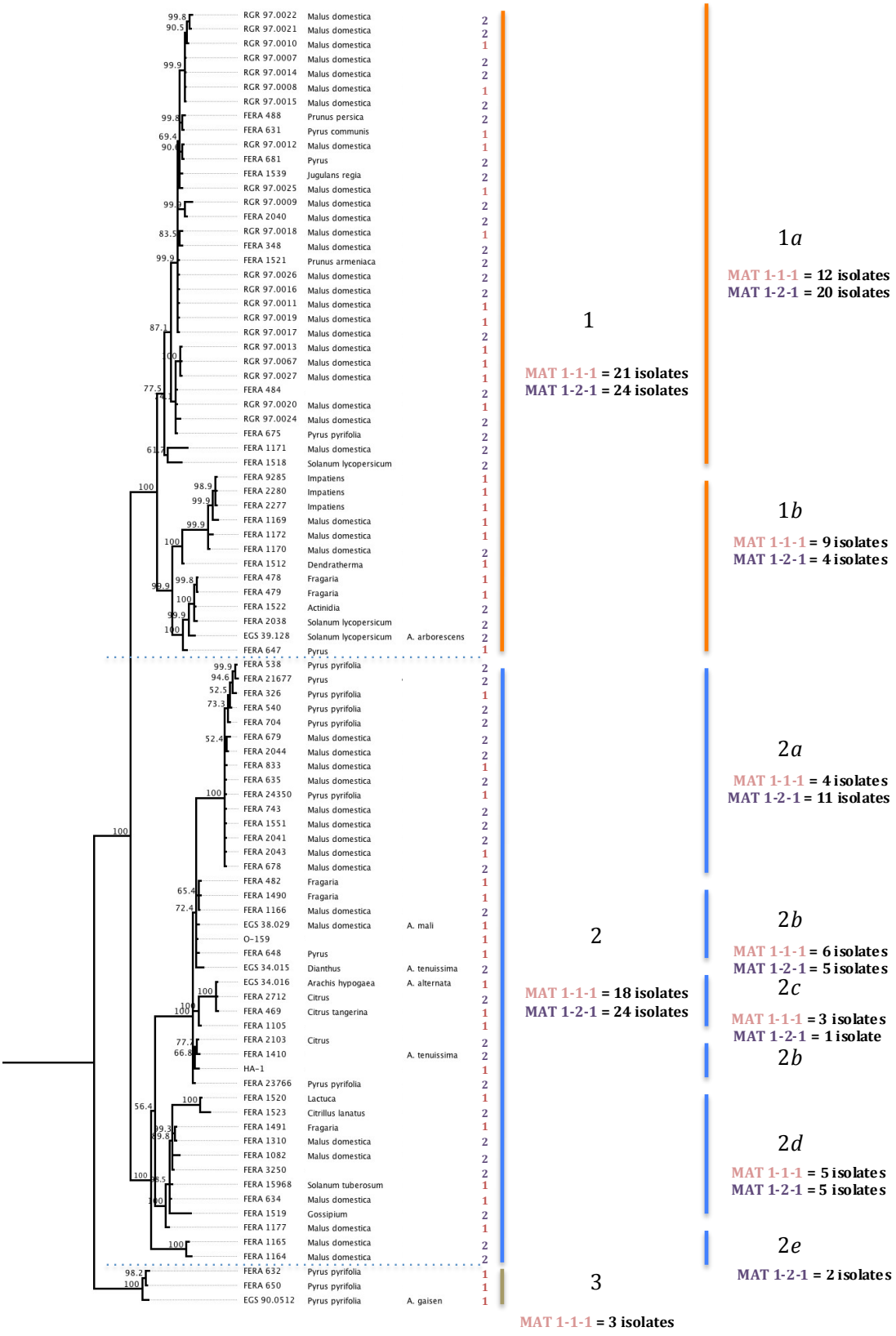


Figure 6.6 Multi-locus phylogeny annotated by isolate mating type: Five-gene phylogeny, as presented in Chapter 3 (Fig. 3.2). Isolates annotated by the mating type gene locus they possess (*MAT1-1-1* or *MAT1-2-1*), as determined by mating type specific PCR.

Identifying genes required for meiosis in genomes of putatively asexual *Alternaria* spp. and sexual Dothideomycetes

The strains of the putatively asexual species *A. alternata* possessed many of the meiotic genes present in *S. cerevisiae* (57 of the 86 meiotic genes tested), including genes in double stranded break generation, synaptonemal complex formation, DNA repair and involved in the resolution crossovers. *A. alternata* possessed homologs for 27 of the 29 core meiotic proteins in eukaryotes, including 10 of the 15 genes that encode meiosis-specific proteins (Table 6.1). 55 meiotic gene homologs were detected in *A. brassicicola* including 24 conserved meiotic genes and seven meiosis-specific genes.

Homologs for the core meiotic genes *Mlh2* and *Hop1* (a meiosis-specific gene) were not identified in the *A. alternata* predicted proteomes, but were also not identified in proteomes of any of the other species. A homolog was not detected for the DNA repair protein *Mei5* in any of the three *A. alternata* proteomes, however a homologue to *Hta1* was detected, which is described as a functional homolog of *Hta2* in the *S. cerevisiae* genome (Appendix, Table 8.2). The profile of meiotic genes in *A. brassicicola* was similar to that of *A. alternata*, however homologs to the core meiotic genes *Rec8*, *Hop2*, *Hop1*, or *Mer3* were not detected.

Sexual Dothideomycetes possessed a similar "profile" of meiotic gene homologs to *Alternaria* spp.: 60 meiotic gene homologs were detected in *P. tritici-repentis* including 27 conserved meiotic genes and 11 meiosis-specific genes; 51 meiotic gene homologs were detected in *C. heterostrophus* including 21 conserved meiotic genes and eight meiosis-specific genes; 54 meiotic gene homologs were detected in *P. nodorum* including 24 conserved meiotic genes and eight meiosis-specific genes (Table 6.1). There were no instances of the three sexual species possessing a meiotic homolog that was not detected in any of the *Alternaria* spp. genomes (Table 6.1).

Table 6.1*a* Homologs of meiotic genes in *Alternaria* spp.: Detection of homologs to 86 genes directly and indirectly involved in meiosis (as identified in Saccharomyces cerevisiae) in *Alternaria* spp. and other Dothideomycetes. Genomes searched were: three strains of *Alternaria alternata* (putatively asexual), *Pyrenophora tritici-repentis* (***Py. tr***: sexual), *Cochliobolus heterostrophus* (***Co. h***: sexual), *Phaeosphaeria nodorum* (***Ph. n***: sexual) and *Alternaria brassicicola* (***Al. b***: putatively asexual). 29 core meiotic genes (CMG) and 15 meiosis-specific genes (MSG) are marked.

Meiotic gene		*Sexual species:*			*Alternaria alternata:*					
Biological process	**Name**	***Py. tr***	***Co. h***	***Ph. n***	***FERA 675***	***FERA 1166***	***FERA 650***	***Al. b***	**CMG**	**MSG**
Double-stranded break generation	***Spo11***	√	.	.	√	√	√	√	**X**	**X**
	Rec107/Mei2	.	.	.	.	.	.	.		
	Mei4	√	√	√	√	√	√	.		**X**
	Rec102	.	.	.	.	.	.	.		
	Rec104	.	.	.	.	.	.	.		
	Rec114	.	.	.	.	.	.	.		
	Ski8	√	√	√	√	√	√	√		
	Mer1	.	.	.	.	.	.	.		
	Hfm1/Mer3	√	√	√	√	√	√	.	**X**	**X**
	Nam8/Mre2	√	√	√	√	√	√	√		
Removal of Spo11	***Mre11***	√	√	√	√	√	√	√	**X**	
	Rad50	√	.	√	√	√	√	√	**X**	
	Xrs2/Nbs1	√	.	.	.	.	.	√		
	Sae2/Com1	√	√	.	√	√	√	√		
Strand invasion	***Rad51***	√	√	√	√	√	√	√	**X**	
	Dmc1	√	.	√	√	√	√	√	**X**	**X**
	Rad52	√	√	√	√	√	√	√	**X**	
	Rad54	√	√	√	√	√	√	√		
	Rdh54	√	√	√	√	√	√	√		
	Rfa1	.	√	√	√	√	√	√		
	Rfa2	√	√	√	√	√	√	√		
	Rfa3	.	.	.	.	.	.	.		
	Sae3	√	.	√	√	√	√	√		**X**
	Rad55	√	√	√	√	√	√	√		
DNA damage checkpoint	***Pch2***	.	.	.	.	.	.	.		
	Mec1	√	√	√	√	√	√	√		
	Rad17	√	√	√	√	√	√	√		
	Rad24	√	√	√	√	√	√	√		
	Ddc1	.	.	.	.	.	.	.		
Regulation of crossover frequency	***Mlh1***	√	√	√	√	√	√	√	**X**	
	Mlh3	√	√	√	√	√	√	√	**X**	
	Msh4	√	.	√	√	√	√	√	**X**	**X**
	Msh5	√	√	√	√	√	√	√	**X**	**X**
	Sgs1	√	√	√	√	√	√	√		
	Mei5	√	√	√	.	.	.	√		**X**
	Mum2	.	.	.	.	.	.	.		
	Ndj1	.	.	.	.	.	.	.		**X**
	Rad1	√	√	√	√	√	√	√	**X**	
	Rad2	√	√	√	√	√	√	√		
Formation of synaptonemal complex	***Hop1***	.	.	.	.	.	.	.	**X**	**X**
	Hop2	√	√	√	√	√	.	.	**X**	**X**
	Mnd1	√	√	.	√	.	√	√	**X**	**X**
	Zip1	.	.	.	.	.	.	.		
	Zip2	.	.	.	.	.	.	.		**X**
	Zip3	.	.	.	.	.	.	.		
	Zip4/Spo22	.	√	.	.	.	.	.		**X**

Table 6.1b Homologs of meiotic genes in *Alternaria* spp.: Detection of homologs to 86 genes directly and indirectly involved in meiosis (as identified in Saccharomyces cerevisiae) in *Alternaria* spp. and other Dothideomycetes. Genomes searched were: three strains of *Alternaria alternata* (putatively asexual), *Pyrenophora tritici-repentis* (***Py. tr***: sexual), *Cochliobolus heterostrophus* (***Co. h***: sexual), *Phaeosphaeria nodorum* (***Ph. n***: sexual) and *Alternaria brassicicola* (***Al. b***: putatively asexual). 29 core meiotic genes (CMG) and 15 meiosis-specific genes (MSG) are marked.

Meiotic gene		*Sexual species:*			*Alternaria alternata:*					
Biological process	**Name**	***Py. tr***	***Co. h***	***Ph. n***	***FERA 675***	***FERA 1166***	***FERA 650***	***Al. b***	**CMG**	**MSG**
DNA repair	***Hta1***	.	.	.	√	√	√	.		
	Hta2	√	√	√	.	.	.	√		
	Red1	.	.	.	.	.	.	.		
	Smc5	√	√	√	√	√	√	√	**X**	
	Smc6	√	√	√	√	√	√	√	**X**	
	Exo1	√	√	√	√	√	√	√		
	Hrr25	√	√	√	√	√	√	.		
	Rad23	√	√	√	√	√	√	√		
Mismatch repair	***Msh2***	√	√	√	√	√	√	√	**X**	
	Msh3	√	√	√	√	√	√	√		
	Msh6	√	√	√	√	√	√	√	**X**	
	Mlh2	.	.	.	.	.	.	.	**X**	
	Pms1	√	√	√	√	√	√	√	**X**	
Resolution of recombination intermediates	***Mms4***	.	.	.	.	.	.	.		
	Mus81	.	.	.	.	.	.	.		
	Slx1	√	√	√	√	√	√	√		
	Top1	√	√	√	√	√	√	√		
	Top2	√	√	√	√	√	√	√		
	Top3	√	√	√	√	√	√	√		
	Slx4	.	.	.	.	.	.	.		
	Slx5	√	.	.	.	.	.	.		
	Slx8	.	.	.	.	.	.	.		
Joining of non-homomogous ends	***Yku70***	√	√	√	√	√	√	√		
	Yku80	√	.	√	√	√	√	√		
	Dnl4	√	√	√	√	√	√	√		
	Lif1	√	.	.	.	.	.	√		
Other	***Msc1***	√	√	√	√	√	√	√		
	Msc7	√	√	√	√	√	√	√		
	Msc3	.	.	.	.	.	.	.		
	Msc6	.	.	.	.	.	.	.		
	Srs2	√	√	√	√	√	√	√		
	Mps3	.	.	.	.	.	.	.		
	Rec8	√	√	.	√	√	√	.	**X**	**X**
	Rad21	√	√	√	√	√	√	√	**X**	
	Smc1	√	.	√	√	√	√	√	**X**	
	Smc2	√	√	√	√	√	√	√	**X**	
	Smc3	√	.	√	√	√	√	√	**X**	
	Smc4	√	√	√	√	√	√	√	**X**	
	Scc3	√	√	√	√	√	√	√	**X**	
	Pds5	√	√	√	√	√	√	√	**X**	
No. gene homologs	**86**	60	51	54	57	56	56	55		
No. core meiotic genes		27	21	24	27	26	26	25	**29**	
No. meiosis specific genes		11	8	8	10	9	9	7		**15**

6.5 DISCUSSION

Detection of recombination in *Alternaria alternata*

In fungal species without directly observed sexual reproduction, indirect methods must be used to infer sexual history and recombination. The presence of mating type genes in phylogenetic clades of the *A. alternata* species group was used as an indication whether population genetic structure was characteristic of a recombining history. Isolates of both *MAT1-1-1* and *MAT1-2-1* were present in major phylogenetic Clades 1 and 2; and were present in six of the eight minor phylogenetic clades representing 85 of 90 isolates (Fig. 6.6). This suggests that recombination has occurred since the formation of these phylogenetic clades. This is in agreement with Berbee *et al.* (2003) who stated that 'because mating type genes themselves cannot have recombined, any characters associated with opposite mating types in a population must have either originated convergently, or been exchanged by recombination'. An example of two pairs of *A. alternata* species group isolates illustrates this.

Isolates *RGR 97.0011* and *RGR 97.0016* (phylogenetic Clade 1*a*, Fig. 6.7) are genetically identical over five loci apart from possessing two opposing *MAT* idiomorphs. Isolates *FERA 2041* and *FERA 2043* (phylogenetic Clade 2*a*, Fig. 6.7) are also genetically identical isolates over five loci apart from possessing two opposing *MAT* idiomorphs. Convergent evolution would suggest that the two isolates carrying the *MAT1-1* idiomorph (*RGR 97.0011* and *FERA 2043*) have evolved from a more recent common ancestor than isolates in the same phylogenetic clade that carry *MAT1-2* idiomorphs (Fig. 6.7: *a*). The two isolates carrying the same multi-locus haplotype in phylogenetic Clade 1*a* differ from the two isolates carrying the same haplotype in Clade 2*a* by 100 SNPs. The alternative explanation is that recombination has occurred between diverging lineages and other populations, leading to the presence of both mating types in the diverging lineage (Fig. 6.7: *b*).

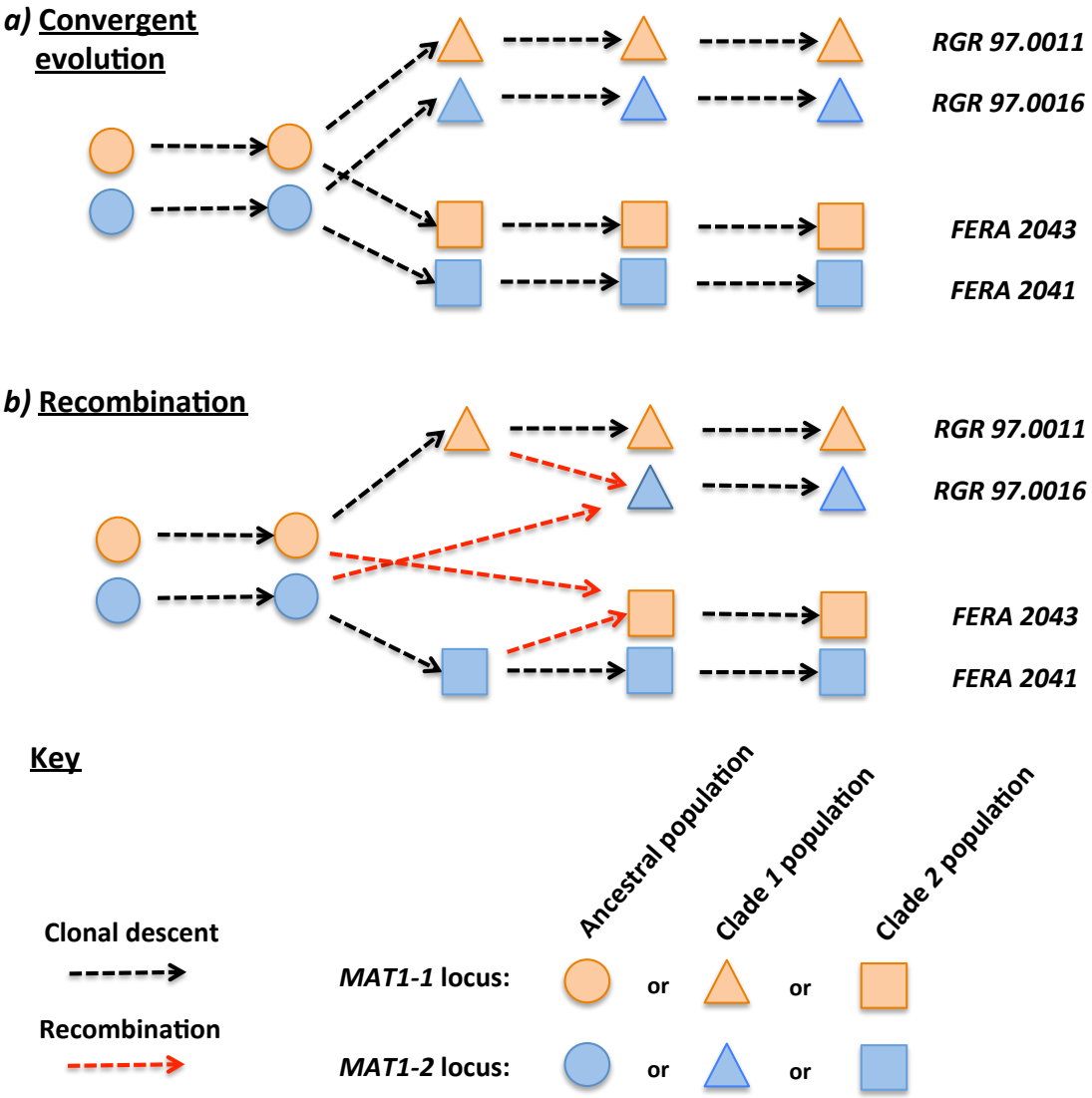


Figure 6.7 Proposed inheritance of mating type loci in *Alternaria alternata*: Descent of individuals in phylogenetic Clade *1* and Clade *2* (Fig. 6.6) from a hypothetical ancestral population. Inheritance is shown assuming a theory of *a*) convergent evolution and *b*) recombination between populations.

This same approach was used to infer that recombination has occurred in *Alternaria* species by Berbee *et al.* (2003). Their study identified that both *MAT1-1* and *MAT1-2* idiomorphs were present in isolates of *A. brassicae*, *A. brassicicola* and *A. alternata* (with an *A. tenuissima* morphology) (Fig. 6.4) based on only two strains from each species (one of each mating type) and the low variability locus ITS. This work has therefore indicated that recombination has occurred on a finer scale than identified in Berbee *et al.* (2003), at a sub-species level in distinct phylogenetic lineages of *A. alternata*. These results are supported by data from 45 isolates within phylogenetic Clade 1 and 42 isolates within phylogenetic Clade 2.

Genealogical concordance species recognition (GSR) identifies species boundaries at the limits of recombination between two operational taxonomic units (Taylor *et al.*, 2000). Identification of recombination within the *A. alternata* species group supports the use of GSR as a technique for species recognition in Chapter 3. Recombination within phylogenetic clades is supported by incongruence between the tree topologies of six single-locus phylogenies (Fig. 3.1: *a-f*), which infer that recombination has occurred within phylogenetic Clades 1 and 2. In this case recombination may be sexual, but it may also be a result of parasexual or other methods of genetic exchange.

Indirect evidence of sexuality in *Alternaria alternata*

This study indicates that the genomes of putatively asexual *Alternaria* spp. possess a compliment of meiotic genes equivalent to that present in related sexual species. Homologs were detected to ten core meiotic genes encoding proteins, which, according to functional annotation studies, only have functions in meiosis (Cherry *et al.*, 2012). 27 of 29 core meiotic genes had homologs in *A. alternata*, with only *Hop1* and *Mlh2* not being detected. These genes may not be essential for successful recombination as *Hop1* has been independently lost on multiple occasions within sexual eukaryotes, with loss in animals (*Drosophila melanogaster*) and in other fungi including the Sodariomycetes (*Neurospora crassa*) and in the Glomeromycota (Malik *et al.*, 2008, Halary *et al.*, 2011). The same is true for *Mlh2*, which has undergone independent losses within the eukaryotes, occurring within both animals and fungi, and has also been shown to be absent in some plants and protists (Ramesh *et al.*, 2005).

Of the 86 genes used in this study, 51-60 had homologs in Dothideomycete species, suggesting that 60%-70% of meiotic genes are conserved between *S. cerevisiae* and this Class. The genes used in this study were previously presented in Halary *et al.* (2011) where homologs of 51 meiotic genes were identified in the putatively asexual *Glomus* species. The detection of similar numbers of homologs to Halary *et al.* (2011) supports the methodology used in this study. The 30-40% meiotic genes without detectable homologs may be present but are too variable for BLAST detection or, if

they are not essential for meiosis, have become pseudogenes and lost from the proteome (Schurko and Logsdon, 2008).

Detection of 27 core meiotic genes and ten meiosis specific genes indicates that *Alternaria* spp. and other Dothideomycetes use a similar model of recombination to that described in Malik *et al.,* (2008; Fig. 6.2), with the exception that *Hop1* may be absent from the synaptonemal complex. By providing the first evidence for presence of an extensive set of meiotic genes in *A. alternata* and *A. brassicicola*, including homologs of meiotic genes this study indicates that putatively asexual *Alternaria* spp. possess the genetic tools for sexuality (Schurko *et al.*, 2009). However, presence of sex specific genes in a genome does not demonstrate that these genes are or expressed or still function in meiosis (Schurko *et al.*, 2009). A model for demonstrated functionality of sex-specific genes has already been established for *A. alternata*, as mating type idiomorphs *MAT1-1* and *MAT1-2* have been cloned and expressed in the sexual Dothideomycete fungus, *Cochlibolus heterostrophus* (Arie *et al.*, 2000). These genes were able to initiate mating between *C. heterostrophus* isolates. Further work is required to confirm if meiotic homologs are functional in *Alternaria* spp..

Mating types being maintained in a 1:1 ratio can be used as evidence that balancing selection is acting on these loci. *MAT* idiomorphs were present in a 1:1 ratio when tested across all 90 *A. alternata* species group isolates shown in Figure 6.5. A 1:1 ratio was observed within major phylogenetic Clade 1 and Clade 2. These results are consistent with frequency-dependent selection occurring (May *et al.*, 1999).

The ratio of mating type idiomorphs has previously been used as evidence of sexuality in haploid, heterothallic fungi: A previous study has investigated the distribution of *MAT* idiomorphs in the heterothallic fungus *Aspergillus fumigatus* (Paoletti *et al.*, 2005). A global collection of *A. fumigatus* isolates was used, in which 43% of isolates carried the *MAT1-1* idiomorph and 57% carried the *MAT1-2* idiomorphs. Similar results have also been reported in the Dothideomycete fungus *Mycosphaerella graminicola* using a worldwide collection of 2035 isolates (Zhan *et al.*, 2002). Both of these studies concluded that detection of a near 1:1 distribution ratio of *MAT* idiomorphs supported evidence of sexual reproduction.

Although two indirect indications of sexuality cannot be used as proof of sexuality (Schurko *et al.*, 2009), this does add to a growing body of indirect evidence in the

Alternaria genus (Linde *et al.*, 2010, Stewart *et al.*, 2011, Stewart *et al.*, 2013). A similar mating type assay was used to investigate the relative abundance of *MAT* idiomorphs in *A. brassicicola* (Fig. 6.4), causing disease on *Cakile maritime*, a sand dune colonising brassicaceous species (Linde *et al.*, 2010). Equal mating type allele frequencies were observed in a total dataset of 210 strains, with 108 isolates possessing the *MAT1-1* locus and 102 isolates possessing the *MAT1-2* locus. These data, along with the identification that microsatellite marker alleles were in linkage equilibrium was considered as evidence of sexuality in *A. brassicicola* populations.

Population studies have shown that *A. alternata* mating type genes are maintained under purifying selection in the environment (Stewart *et al.*, 2011). Purifying selection was suggested to be the result of cryptic sexuality, a recent sexual past or the involvement of *MAT* genes in other critical cellular functions. The identification of homologs to ten meiosis-specific genes in *Alternaria* spp. genomes will allow future work to investigate whether these show evidence of purifying selection. This would offer strong indirect evidence of sexuality in the *Alternaria* genus.

Possible mechanisms of genetic exchange occurring within an *A. alternata* citrus grove population were investigated recently (Stewart *et al.*, 2013). 50 isolates were collected that formed three sub-populations. All three sub-populations were observed to be predominantly asexual. However two of these populations contained both *MAT* idiomorphs and showed signs of genetic recombination. The third sub-population comprised a single *MAT* idiomorph but still exhibited genetic signatures of recombination. This was suggested to be a result of parasexual processes occurring within the sub-population. Stewart *et al.* (2013) suggested that both sexual and parasexual processes may be occurring within *A. alternata* on citrus. Sexuality and parasexuality have been shown to co-occur in other fungi including *Magnaporthe oryzae* (Noguchi *et al.*, 2006), *Aspergillus nidulans* (Schoustra *et al.*, 2007) and *Cryphonectria parasitica* (Milgroom *et al.*, 2009).

Parasexual processes have previously been suggested for *A. alternata*. Horizontal gene transfer (HGT) has repeatedly been speculated as a mechanism for acquisition of a conditionally dispensable chromosome (CDC) that confers pathogenicity to particular hosts (Tanaka *et al.*, 1999, Hatta *et al.*, 2002, Masunaka *et al.*, 2005, Lawrence *et al.*, 2008, Akagi *et al.*, 2009, Mehrabi *et al.*, 2011, Hu *et al.*, 2012)

(Described in detail in Chapter 5). It is possible that genes involved in meiotic recombination that have been identified in this study have been conserved to perform functions in parasexual recombination (Halary *et al.*, 2011). An isolate of *A. alternata* has been identified that is pathogenic to both tangerine and rough lemon and possesses multiple CDCs (Masunaka *et al.*, 2005). This isolate was speculated to have arisen by parasexual processes, possibly through the anastomosis of an *A. alternata* tangerine pathotype and an *A. alternata* rough lemon pathotype.

Further evidence is needed before sexuality can be confidently determined to occur in *A. alternata*. Genome sequencing data generated for other fungal species has allowed deeper investigation into the occurrence of sexuality in those systems. This has included the detection of RIP in transposon repeats (Ropars *et al.*, 2012), and finding that pheromone precursor and receptor genes are constitutively expressed in culture (Poggeler, 2002, Paoletti *et al.*, 2005). Generation of genomic sequence data for *A. alternata* isolates offers the chance to assess whether similar evidence can be found in *Alternaria* spp..

Implications of recombination

This work does not provide direct proof of sexuality in the *A. alternata* species group. However, it does provide strong evidence for recombination. Taylor *et al.* (1999) considered that without direct observation, studies cannot determine conclusively whether recombination is occurring by sexual or parasexual processes in fungi, or how often it is occurring. However, the evidence for the current assumption of asexuality in the *Alternaria* genus is based on the lack of observation of a teleomorphic state. Infrequent mating may occur cryptically and still offer genetic benefits (Schurko *et al.*, 2009). *A. alternata* are ubiquitous saprophytes and found on a diverse range of plant and animal hosts; recombination, leading to novel combinations of beneficial alleles subject to natural selection at a higher efficacy (Goddard *et al.*, 2005), may be responsible for the adaptation of this group to diverse environments (Taylor *et al.*, 1999) and plant hosts (Linde *et al.*, 2010).

Alternaria mating type primers are suitable for multiplex

All fungal isolates tested carried one of the two mating type genes. This confirmed the *A. alternata* species group as heterothallic fungi as proposed by Arie *et al.* (2000). The mating type PCR reaction was performed in multiplex, using *MAT1-1-1* gene (*AAM1-2*) and *MAT1-2-1* gene (*AAM1-3*) primers designed by (Arie *et al.*, 2000). This methodology halved the number of PCR reactions that had to be performed when identifying an isolates mating type. This provided savings in time and cost of reagents. Multiplex PCR has been used to detect mating types in other plant pathogens including *Aspergillus fumigatus* (Paoletti *et al.*, 2005) and *Tapasia yallundae* (Dyer *et al.*, 2001), *Ascochyta lentis* (Cherif *et al.*, 2006) and in *A. brassicicola* (Linde *et al.*, 2010). Multiplex PCR has not previously been used in studies detecting mating type in *A. alternata* (Arie *et al.*, 2000, Akagi *et al.*, 2009, Stewart *et al.*, 2011, Stewart *et al.*, 2013). This methodology is therefore suitable for future mating type screens within *Alternaria*.

CHAPTER 7

GENERAL DISCUSSION

This study aimed to characterise the *Alternaria alternata* species group with particular focus on the causal agent of apple leaf blotch and pear black spot, which are of particular phytosanitary concern to the UK and Europe. This work complements the recent reclassifications of the wider *Alternaria* genus and related species (Fig. 1.2; Lawrence *et al.*, 2013, Woudenberg *et al.*, 2013).

Evolutionary relationships within the *A. alternata* species group were established using a phylogenetic approach based on highly variable functional genes (Chapter 3). This is the first time that the principles of genealogical concordance species recognition (GSR) have been used to delimit lineages within the *A. alternata*. Using these approaches three major lineages were identified in the *A. alternata* species group. Incongruity was identified between tree topologies of different loci with support for three phylogenetically distinct lineages (Fig. 7.1). These findings indicate that there are currently more morphological species within the *A. alternata* than can be supported under a phylogenetic framework. This was shown demonstrated for representative strains used in species descriptions for *A. tenuissima*, *A. alternata* and *A. mali* (Simmons, 2007), which were shown to represent the same genetic taxon.

Morphological variation within the *A. alternata* was also studied (Chapter 4). Analysis of sporulation patterns showed that nine morphological groups could be identified within the *A. alternata* isolates used in this study. Spore characters associated with these groups were considered in the context of morphological species descriptions by Simmons and Roberts (1993) and Simmons (2007). Four morphological groups were identified that showed spore characters associated with the described morphological species *A. arborescens* and these were found to be associated with a single phylogenetic clade (Clade 1; Fig. 7.1). The five other morphological groups identified in this study had characters associated with the described morphologically similar species *A. tenuissima* and *A. mali* and were associated with phylogenetic Clade 2. Characters associated with *A. alternata*, a morphotype rarely found in nature (Simmons, 1999b) were also associated with phylogenetic Clade 2 (Fig. 7.1).

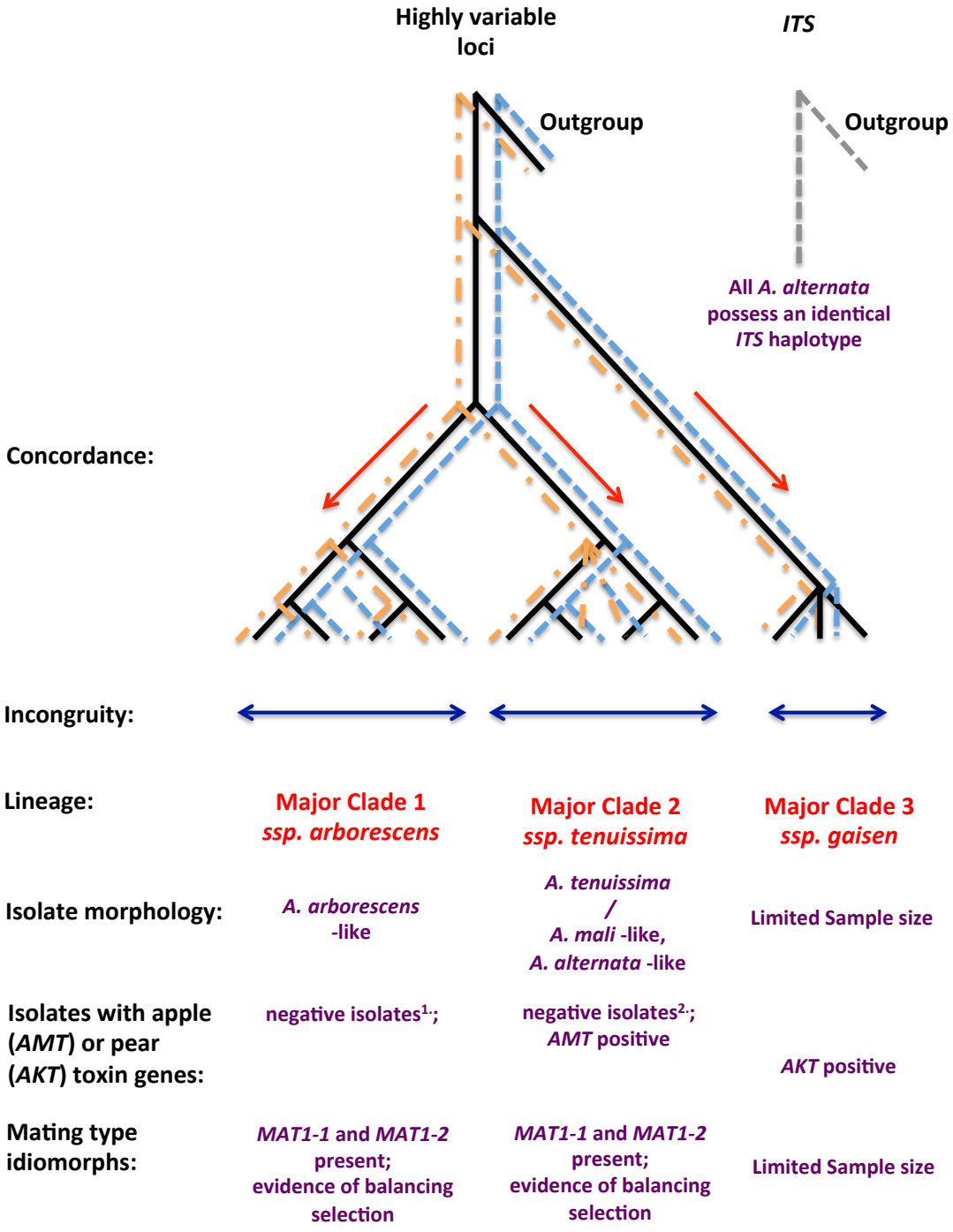


Figure 7.1 Summary of findings from characterisation of the *Alternaria alternata* species group: Findings are annotated onto an idealised multi-locus phylogeny. All *A. alternata* species group isolates carry the same ITS haplotype, this can be used to identify all isolates as members of the species *A. alternata*. Below this, highly variable regions support the evolution of three distinct lineages within the *A. alternata*. Incongruity between tree topologies marks population level variation within these lineages. Lineages are named as subspecies, with names deemed appropriate from evidence of the morphological characters associated with these lineages and the detection of toxin synthesis genes in isolates. A limited sample size of three isolates limits conclusions that could be drawn in ssp. *gaisen*.

[1.] Isolates within this clade were isolated from lesions on apple leaves, despite testing negative for apple toxin genes. [2.] Experimental work on isolates in this clade showed that only isolates possessing apple toxin genes were pathogenic on apple leaves.

This is the first time that such a clear link has been found between the morphology of *Alternaria* isolates and their position in a phylogeny. As such the phylogenetic lineages (Clades 1 and 2) were named as *A. alternata* ssp. *arborescens* and *A. alternata* ssp. *tenuissima*. The current morphological descriptions for *A. arborescens* and *A. tenuissima* as presented in Simmons (2007) should be used for these subspecies, and representative isolates *EGS 39.128* and *EGS 34.016* should be designated as the type isolates respectively. These subspecies are also representative of sporulation groups 3 and 5 as described in Simmons and Roberts (1993; Fig. 4.2).

Isolates from phylogenetic Clade 3 could not be morphologically characterised as only one isolate from this clade was included in the analysis. The clade contained the representative isolate for the described morphological species *A. gaisen* (*EGS 90. 0512*; Simmons (2007)) and as such was named *A. alternata* ssp. *gaisen*. Further morphological analysis is required to establish variation within this lineage but it is recommended that *EGS 90.0512* should be designated as the current type isolate. This subspecies is also expected to be representative of sporulation group 2 as presented in Simmons and Roberts (1993; Fig. 4.2).

The presence of *AMT* (apple toxin) and *AKT* (pear toxin) genes in apple and pear pathotypes, was determined within *A. alternata* isolates (Fig. 7.1). Kusaba and Tsuge (1995b) showed that the *A. alternata* species group contains seven *A. alternata* pathotypes (apple, pear, tomato, strawberry, rough-lemon, tangerine and tobacco), but could not resolve these pathotypes phylogenetically. This is the first time distribution of toxin genes have been investigated within a phylogeny of the *A. alternata* species group (Chapter 5). No isolates in the ssp. *arborescens* lineage (Clade 1) possessed the genes required for AMT or AKT production. However, sequencing of an *A. alternata* tomato pathotype (*EGS 38.128*; Hu *et al.* (2012)) showed that isolates in this lineage are capable of possessing CDCs (Fig. 3.2: Clade 1*b*). The ssp. *tenuissima* lineage (Clade 2) was the only clade to contain isolates that possessed the genes required for AMT synthesis. The ssp. *gaisen* lineage (Clade 3) exclusively consisted of isolates *ex.* pear that possessed genes required for AKT production. Further investigation is required to establish whether ssp. *gaisen* (Clade 3) is a lineage of pear specific pathogens.

Findings from this work supported the use of a dual nomenclature system to designate isolates as pathotypes separate from their species names. If some but not all isolates in ssp. *tenuissima* possess AMT synthesis genes on CDCs then genes on essential chromosomes (ECs) cannot be used to determine whether isolates are apple pathotypes or not.

Results from this work raised further questions, as isolates in the ssp. *arborescens* clade that have been reported to cause disease on apple leaf tested negative for the genes required for AMT synthesis. This questions the concept that AMT production is the sole determinant of whether an isolate can successfully cause disease on apple leaf. Confidence is shown in toxin production being responsible for pathogenicity in isolates within the ssp. *tenuissima* lineage (Clade 2) as isolates that tested positive for genes required for AMT synthesis (13-15 *AMT* genes) were shown to be pathogenic on apple leaves, whereas isolates that did not possess these HST-genes were not pathogenic. Isolates from ssp. *arborescens* (Clade 1) were not tested for pathogenicity in this study, and Koch's postulates have not been completed on these isolates. Further investigation is required to identify whether ssp. *arborescens* isolates are pathogenic and if so, what factors are involved in their pathogenicity.

PCR screens for HST-genes showed presence of secondary banding and also indicated that isolates could possessed some, but not all genes required for HST synthesis. This led to the conclusion that although primers designed by Johnson *et al.* (2000b) and Roberts *et al.* (2011) were useful in indicating presence and distribution of apple and pear pathotype for some isolates in this study, their unreliability meant that they were not suitable for routine identification of *A. alternata* pathotypes. Novel molecular markers specific to regions of CDCs should be designed for identification of *A. alternata* apple and pear pathotypes. Furthermore, due to some isolates testing positive for incomplete sets of toxin genes required for AMT or AKT synthesis these assays should be based upon multiple regions within the toxin gene cluster or to other, more conserved, regions of the CDC.

Table 7.1 Diversity of characters associated with the 12 *Alternaria alternata* isolates used for genome sequencing: Genome sequencing data represents a resource allowing further investigation of *A. alternata* isolates from different phylogenetic lineages, morphological characters, representing apple (*AMT* positive) and pear (*AKT* positive) pathotypes, non-pathotypes (*AMT*, *AKT*, *AFT*, *ACTT*, *ACRT*, *ALT* negative) and representing both mating type idiomorphs.

Isolate	Phylogenetic clade (Fig. 3.7)	Morphological clade (Fig. 4.6)	Toxin genes (Table 5.6): *AMT*	*AKT*	Mating type idiomorph (Fig. 6.5)
FERA 675 *ex.* pear	Clade 1	*arborescens*-like (G)			*MAT 1-2-1*
RGR 97.0013 *ex.* apple		*arborescens*-like (B)			*MAT 1-1-1*
RGR 97.0016 *ex.* apple		*alternata*-like (C)			*MAT 1-2-1*
FERA 648 *ex.* pear	Clade 2	*tenuissima*-like (A)			*MAT 1-1-1*
FERA 1082 *ex.* apple		*tenuissima*-like (E)			*MAT 1-2-1*
FERA 1164 *ex.*apple		*tenuissima*-like (A)			*MAT 1-2-1*
FERA 24350 *ex.* pear		*tenuissima*-like (A)			*MAT 1-1-1*
FERA 635 *ex.* apple		*tenuissima*-like (A)	√		*MAT 1-2-1*
FERA 743 *ex.* apple		n/a	√		*MAT 1-2-1*
FERA 1166 *ex.* apple		*tenuissima*-like (E)	√		*MAT 1-2-1*
FERA 1177 *ex.* apple		n/a	√		*MAT 1-1-1*
FERA 650 *ex.* pear	Clade 3	*tenuissima*-like (A)		√	*MAT 1-1-1*

During the course of this study genome sequence data was generated for a diverse set of 12 *A. alternata* isolates (Table 7.1). Further bioinformatic work needs to be performed: accuracy of gene models needs to be assessed, gene prediction is required for the remaining genomes and functional annotation should be performed on genes. These genomes constitute a valuable resource that will allow deeper investigation into the evolution of *A. alternata*. Recent genomic analyses by other researchers show how whole genome sequence data can be used in future studies: Hu *et al.* (2012) recently identified and characterised the CDC from the *A. alternata* tomato pathotype. Origin of CDCs and occurrence of horizontal gene transfer can be investigated using comparative genomics similar to Richards *et al.* (2011) who showed that oomycete secreted proteins were acquired from fungi. Recombination and sexuality can be further investigated through identification of repeat induced point mutation, as performed in *Penicillium roqueforti* (Ropars *et al.*, 2012), or through analysis of transcriptome data to show constitutive expression of sex pheromones and receptors, as observed in *Aspergillus fumigatus* (Poggeler, 2002). Immediate objectives should focus on sequencing a genome for each of the seven *A. alternata* pathotypes: apple

(four strains sequenced, this study), pear (one strain sequenced, this study), tomato (one strain sequenced, Hu *et al.* (2012)), strawberry, rough lemon, tangerine and tobacco (unsequenced). Through comparative genomics the structure of the chromosomes can be characterised. Similarities in structure of chromosomes can be used to infer when and how they were acquired (testing hypotheses suggested in Fig. 5.11). This would also have practical application to management of *A. alternata*. The design of novel primers in Chapter 3 highlighted how whole genome sequence data can be used to generate phylogenetically informative molecular markers, and as such these genomes can be used to develop diagnostics for specific identification of *A. alternata* pathotypes in accordance with barcoding initiatives for EU quarantine organisms (Bonants *et al.*, 2010).

The presence of different mating type genes in *A. alternata* isolates was used to assess evidence for recombination and potential sexuality (Chapter 6). Evidence was found, as both *MAT* idiomorphs have been inherited into the ssp. *arborescens* lineage (Clade 1) and into the ssp. *tenuissima* lineage (Clade 2; Fig. 7.1). *A. alternata* genomes were shown to possess an equivalent compliment of meiotic genes to sexual Dothideomycetes, providing the first evidence that this species is capable of recombination. Additional analysis of the *MAT* data contributed to a growing body of evidence for a recent sexual past or current cryptic sexuality within the *A. alternata,* as *MAT* idiomorphs were present in a 1:1 ratios indicating that balancing selection is occurring within these ssp. *tenuissima* and ssp. *arborescens*. If sexuality or recombination is still occurring in *A. alternata* then recombination may lead to the generation of progeny with new combinations of alleles, possibly allowing adaptation to new habitats or hosts.

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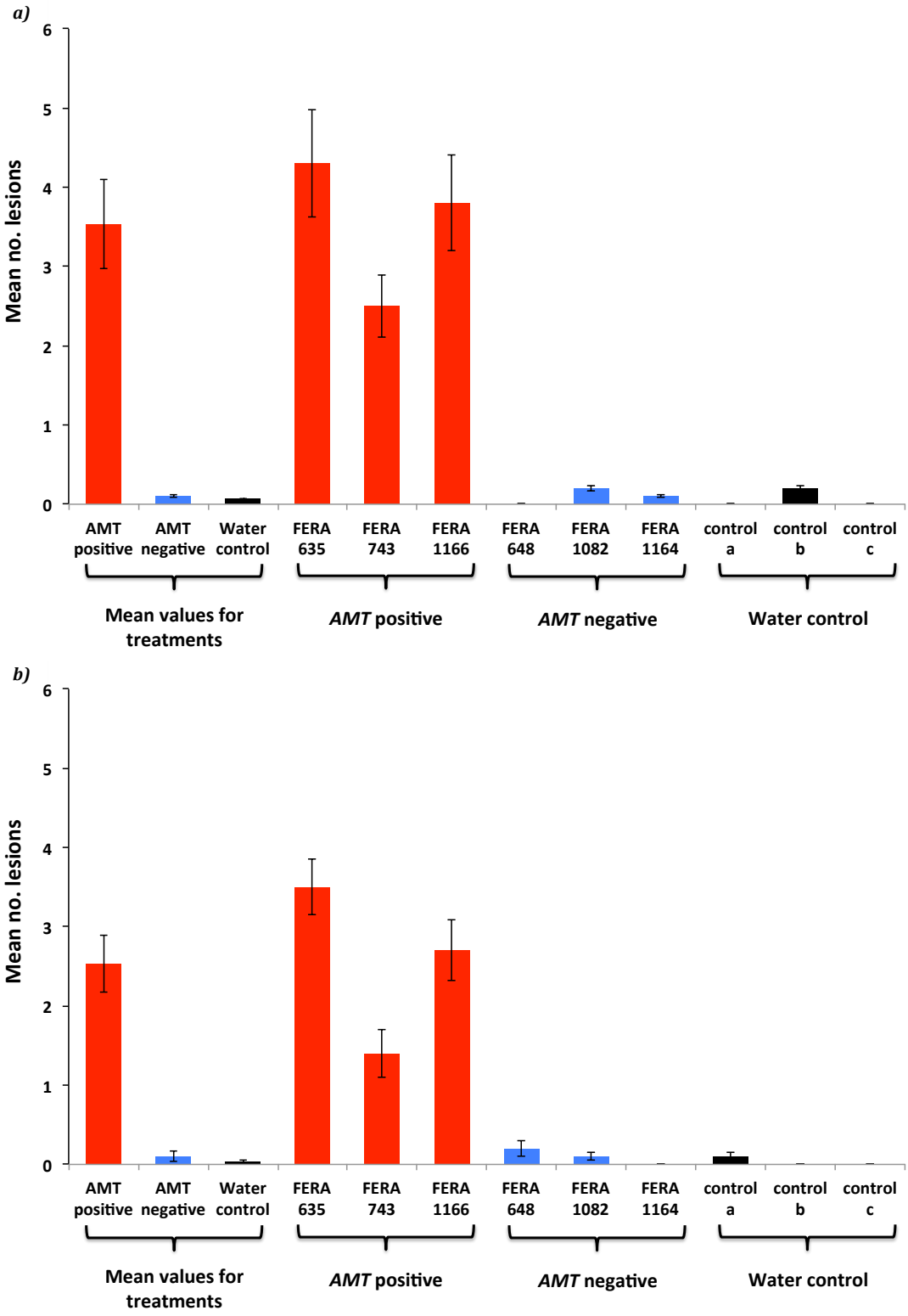
APPENDIX



Supplementary figure 8.1 Mean number of lesions per leaf for treatments in virulence assays: On *a) cv.* Spartan or *b) cv.* Bramley's seedling leaves infected with *Alternaria alternata* isolates possessing *AMT* toxin genes, not possessing *AMT* toxin genes or water. Mean number of lesions (±SE) as observed at 14 dpi. Results are shown for each treatment and isolates nested within each treatment.

Supplementary table 8.1*a* Results from BLASTn searches of 40 toxin synthesis genes against the genome of *FERA 635*: Toxin genes are ordered by their associated pathotype and display the homolog group they belong to (*a*-*e*). The super-contigs that BLAST alignments were made to are shown, including their mean coverage by sequence data during genome assembly. Alignment details are also shown, including the % of the query sequence comprising the alignment and the % of identical of sites within alignments.

FERA 635

BLAST Queery			Homolog group	Similarity in contig:			Alignment details		
Pathotype	Gene	Length		Contig	Length	Coverage	% Query sequence	E-value	% Identity
Apple	*AMT1*	13092		404	14012	52	81	0	99
	AMT2	1254	*a*	432	2604	69	100	0	100
	AMT3	1561		569	3712	73	100	0	99
	AMT4	913		2	13886	73	100	0	97
	AMT5	1223		2	13886	73	100	0	99
	AMT6	1403		2	13866	73	100	0	97
	AMT7	1797		2	13886	73	100	0	98
	AMT8	2829		2	13886	73	100	0	99
	AMT9	1411		2	13886	73	88	0	97
	AMT10	2703		660	4423	37	100	0	99
	AMT11	1008		1824	13273	36	95	$1.6x10^{-135}$	72
	AMT12	1248		1824	13273	36	100	0	97
	AMT13	497		717	9912	56	100	0	100
	AMT14	1105		717	9912	56	100	0	99
	AMT15	555							
	AMT16	1183		717	9912	56	100	0	99
	AMTR1	2461		740	9824	56	100	0	95
Pear	*AKT1*	2028	*b*						
	AKT2	849	*c*						
	AKT3	1061	*d*						
	AKTR	1335	*e*						
Strawberry	*AFT1-1*	2027	*b*						
	AFT3-1	1074	*d*						
	AFT3-2	1075	*d*						
	AFT9-1	9073							
	AFT10-1	1843		1411	31500	37	7	$5.4x10^{-18}$	77
	AFT11-1	1746							
	AFT12-1	894							
	AFTS1	1252	*a*	432	2604	69	96	0	77
	AFTR-1	1338	*e*						
	AFTR-2	1335	*e*						
Tangerine	*ACTT1*	785	*b*						
	ACTT2	849	*c*						
	ACTT3	1061	*d*						
	ACTTR	1308	*e*						
	ACTT5	1883							
	ACTT6	897							
Rough lemon	*ACRTS1*	1394		1562	28486	19	80	$8.3x10^{-46}$	65
	ACRTS2	7958							
Tomato	*ALT1*	668							

Supplementary table 8.1*b* Results from BLASTn searches of 40 toxin synthesis genes against the genome of *FERA 648*: Toxin genes are ordered by their associated pathotype and display the homolog group they belong to (*a*-*e*). The super-contigs that BLAST alignments were made to are shown, including their mean coverage by sequence data during genome assembly. Alignment details are also shown, including the % of the query sequence comprising the alignment and the % of identical of sites within alignments.

FERA 648

BLAST Queery			Homolog group	Similarity in contig:			Alignment details		
Pathotype	Gene	Length		Contig	Length	Coverage	% Query sequence	E-value	% Identity
Apple	*AMT1*	13092							
	AMT2	1254	*a*	48	731256	40	25	$8.7x10^{-26}$	69
	AMT3	1561							
	AMT4	913							
	AMT5	1223							
	AMT6	1403							
	AMT7	1797		88	392709	40	11	$2.6x10^{-15}$	70
	AMT8	2829							
	AMT9	1411							
	AMT10	2703							
	AMT11	1008		107	9756	42	6	$1.5x10^{-21}$	100
	AMT12	1248							
	AMT13	497							
	AMT14	1105							
	AMT15	555		1549	740766	39	51	$9.3x10^{-34}$	72
	AMT16	1183		155	682085	40	100	0	89
	AMTR1	2461		389	982039	39	97	0	70
Pear	*AKT1*	2028	*b*						
	AKT2	849	*c*						
	AKT3	1061	*d*						
	AKTR	1335	*e*						
Strawberry	*AFT1-1*	2027	*b*						
	AFT3-1	1074	*d*						
	AFT3-2	1075	*d*						
	AFT9-1	9073							
	AFT10-1	1843							
	AFT11-1	1746							
	AFT12-1	894							
	AFTS1	1252	*a*	269	587524	39	7	$3.9x10^{-11}$	77
	AFTR-1	1338	*e*						
	AFTR-2	1335	*e*						
Tangerine	*ACTT1*	785	*b*						
	ACTT2	849	*c*						
	ACTT3	1061	*d*						
	ACTTR	1308	*e*						
	ACTT5	1883							
	ACTT6	897							
Rough lemon	*ACRTS1*	1394		157	3999541	40	80	$7.9x10^{-46}$	65
	ACRTS2	7958							
Tomato	*ALT1*	668							

Supplementary table 8.1*c* Results from BLASTn searches of 40 toxin synthesis genes against the genome of *FERA 650*: Toxin genes are ordered by their associated pathotype and display the homolog group they belong to (*a*-*e*). The super-contigs that BLAST alignments were made to are shown, including their mean coverage by sequence data during genome assembly. Alignment details are also shown, including the % of the query sequence comprising the alignment and the % of identical of sites within alignments.

FERA 650

BLAST Queery			Homolog group	Similarity in contig:			Alignment details		
Pathotype	Gene	Length		Contig	Length	Coverage	% Query sequence	E-value	% Identity
Apple	*AMT1*	13092							
	AMT2	1254	*a*	108	310	25	25	$2.5x10^{-26}$	69
	AMT3	1561							
	AMT4	913							
	AMT5	1223							
	AMT6	1403							
	AMT7	1797		429	620256	25	11	$2.5x10^{-13}$	69
	AMT8	2829							
	AMT9	1411							
	AMT10	2703							
	AMT11	1008		739	14799	26	8	$2.3x10^{-19}$	88
	AMT12	1248							
	AMT13	497							
	AMT14	1105		150	7636	126	91	0	85
	AMT15	555		1167	38302	24	47	$1.6x10^{-24}$	70
	AMT16	1183		126	201531	25	100	0	89
	AMTR1	2461		150	7636	125	100	0	87
Pear	*AKT1*	2028	*b*	232	4895	196	100	0	100
	AKT2	849	*c*	135	3527	227	55	0	100
	AKT3	1061	*d*	112	5058	210	100	0	100
	AKTR	1335	*e*	135	3527	227	90	0	100
Strawberry	*AFT1-1*	2027	*b*	232	4895	196	100	0	95
	AFT3-1	1074	*d*	112	5058	211	100	0	95
	AFT3-2	1075	*d*	112	5058	211	100	0	95
	AFT9-1	9073		373	5086	70	56	0	97
	AFT10-1	1843		478	1322	67	72	0	97
	AFT11-1	1746		478	4659	67	100	0	96
	AFT12-1	894		193	12521	48	100	0	86
	AFTS1	1252	*a*	67	366378	25	7	$3.2x10^{-12}$	78
	AFTR-1	1338	*e*	135	3527	227	90	0	97
	AFTR-2	1335	*e*	135	3527	227	90	0	97
Tangerine	*ACTT1*	785	*b*	285	14791	89	108	0	80
	ACTT2	849	*c*	232	4895	195	92	0	90
	ACTT3	1061	*d*	112	5058	211	100	0	95
	ACTTR	1308	*e*	135	3527	227	92	0	94
	ACTT5	1883		193	12521	48	100	0	91
	ACTT6	897		90	2092	97	98	0	92
Rough lemon	*ACRTS1*	1394		93	6066917	25	80	$1.7x10^{-41}$	64
	ACRTS2	7958							
Tomato	*ALT1*	668							

Supplementary table 8.1*d* Results from BLASTn searches of 40 toxin synthesis genes against the genome of *FERA 675*: Toxin genes are ordered by their associated pathotype and display the homolog group they belong to (*a*-*e*). The super-contigs that BLAST alignments were made to are shown, including their mean coverage by sequence data during genome assembly. Alignment details are also shown, including the % of the query sequence comprising the alignment and the % of identical of sites within alignments.

FERA 675

BLAST Queery			Homolog group	Similarity in contig:			Alignment details		
Pathotype	Gene	Length		Contig	Length	Coverage	% Query sequence	E-value	% Identity
Apple	*AMT1*	13092							
	AMT2	1254	*a*	419	311	27	25	$2.0x10^{-27}$	70
	AMT3	1561							
	AMT4	913							
	AMT5	1223							
	AMT6	1403							
	AMT7	1797		189	557392	28	11	$1.1x10^{-13}$	69
	AMT8	2829							
	AMT9	1411							
	AMT10	2703							
	AMT11	1008		3309	57	28	6	$1.5x10^{-21}$	100
	AMT12	1248							
	AMT13	497							
	AMT14	1105							
	AMT15	555		268	2850	187	50	$3.3x10^{-33}$	71
	AMT16	1183		137	263735	27	100	0	89
	AMTR1	2461		385	810237	27	97	0	69
Pear	*AKT1*	2028	*b*						
	AKT2	849	*c*						
	AKT3	1061	*d*						
	AKTR	1335	*e*						
Strawberry	*AFT1-1*	2027	*b*						
	AFT3-1	1074	*d*						
	AFT3-2	1075	*d*						
	AFT9-1	9073							
	AFT10-1	1843							
	AFT11-1	1746							
	AFT12-1	894							
	AFTS1	1252	*a*	358	1488139	28	7	$7.4x10^{-14}$	79
	AFTR-1	1338	*e*						
	AFTR-2	1335	*e*						
Tangerine	*ACTT1*	785	*b*						
	ACTT2	849	*c*						
	ACTT3	1061	*d*						
	ACTTR	1308	*e*						
	ACTT5	1883							
	ACTT6	897							
Rough lemon	*ACRTS1*	1394		1269	250083	28	80	$2.0x10^{-53}$	65
	ACRTS2	7958							
Tomato	*ALT1*	668							

Supplementary table 8.1*e* Results from BLASTn searches of 40 toxin synthesis genes against the genome of *FERA 743*: Toxin genes are ordered by their associated pathotype and display the homolog group they belong to (*a*-*e*). The super-contigs that BLAST alignments were made to are shown, including their mean coverage by sequence data during genome assembly. Alignment details are also shown, including the % of the query sequence comprising the alignment and the % of identical of sites within alignments.

FERA 743

BLAST Queery			Homolog group	Similarity in contig:			Alignment details		
Pathotype	Gene	Length		Contig	Length	Coverage	% Query sequence	E-value	% Identity
Apple	*AMT1*	13092		506	6626	96	51	0	100
	AMT2	1254	*a*	380	2604	141	100	0	100
	AMT3	1561		502	2428	135	100	0	99
	AMT4	913		95	5435	127	100	0	97
	AMT5	1223		74	8427	128	100	0	99
	AMT6	1403		74	8427	128	100	0	97
	AMT7	1797		74	8427	128	100	0	98
	AMT8	2829		74	8427	128	85	0	99
	AMT9	1411		95	5435	127	88	0	97
	AMT10	2703		349	14246	63	100	0	99
	AMT11	1008		6682	21209	51	95	$1.6x10^{-44}$	72
	AMT12	1248		6682	21209	51	100	0	97
	AMT13	497		1101	13851	64	100	0	100
	AMT14	1105		1101	13851	64	100	0	99
	AMT15	555		462	5210	130	57	$4.8x10^{-44}$	70
	AMT16	1183		1101	13851	64	101	0	98
	AMTR1	2461		349	14246	63	100	0	95
Pear	*AKT1*	2028	*b*						
	AKT2	849	*c*						
	AKT3	1061	*d*						
	AKTR	1335	*e*						
Strawberry	*AFT1-1*	2027	*b*						
	AFT3-1	1074	*d*						
	AFT3-2	1075	*d*						
	AFT9-1	9073							
	AFT10-1	1843		1786	53457	58	7	$5.3x10^{-18}$	77
	AFT11-1	1746							
	AFT12-1	894							
	AFTS1	1252	*a*	380	2604	141	96	0	77
	AFTR-1	1338	*e*						
	AFTR-2	1335	*e*						
Tangerine	*ACTT1*	785	*b*						
	ACTT2	849	*c*						
	ACTT3	1061	*d*						
	ACTTR	1308	*e*						
	ACTT5	1883							
	ACTT6	897							
Rough lemon	*ACRTS1*	1394		924	262078	31	80	$8.4x10^{-46}$	65
	ACRTS2	7958							
Tomato	*ALT1*	668							

Supplementary table 8.1*f* Results from BLASTn searches of 40 toxin synthesis genes against the genome of *FERA 1082*: Toxin genes are ordered by their associated pathotype and display the homolog group they belong to (*a*-*e*). The super-contigs that BLAST alignments were made to are shown, including their mean coverage by sequence data during genome assembly. Alignment details are also shown, including the % of the query sequence comprising the alignment and the % of identical of sites within alignments.

FERA 1082

BLAST Queery			Homolog group	Similarity in contig:			Alignment details		
Pathotype	Gene	Length		Contig	Length	Coverage	% Query sequence	E-value	% Identity
Apple	*AMT1*	13092							
	AMT2	1254	*a*	294	125526	16	25	$8.8x10^{-26}$	69
	AMT3	1561							
	AMT4	913							
	AMT5	1223							
	AMT6	1403							
	AMT7	1797		114	116704	16	11	$1.1x10^{-13}$	69
	AMT8	2829							
	AMT9	1411							
	AMT10	2703							
	AMT11	1008		430	111740	17	6	$1.5x10^{-21}$	100
	AMT12	1248							
	AMT13	497							
	AMT14	1105							
	AMT15	555		999	74050	32	57	$4.6x10^{-44}$	70
	AMT16	1183		612	34610	16	100	0	89
	AMTR1	2461		239	209276	16	97	0	69
Pear	*AKT1*	2028	*b*						
	AKT2	849	*c*						
	AKT3	1061	*d*						
	AKTR	1335	*e*						
Strawberry	*AFT1-1*	2027	*b*						
	AFT3-1	1074	*d*						
	AFT3-2	1075	*d*						
	AFT9-1	9073							
	AFT10-1	1843		527	10927	34	8	$3.3x10^{-14}$	72
	AFT11-1	1746							
	AFT12-1	894							
	AFTS1	1252	*a*	873	92962	16	7	$7.7x10^{-14}$	79
	AFTR-1	1338	*e*						
	AFTR-2	1335	*e*						
Tangerine	*ACTT1*	785	*b*						
	ACTT2	849	*c*						
	ACTT3	1061	*d*						
	ACTTR	1308	*e*						
	ACTT5	1883							
	ACTT6	897							
Rough lemon	*ACRTS1*	1394		213	197441	16	80	$8.0x10^{-46}$	65
	ACRTS2	7958							
Tomato	*ALT1*	668							

Supplementary table 8.1*g* Results from BLASTn searches of 40 toxin synthesis genes against the genome of *FERA 1164*: Toxin genes are ordered by their associated pathotype and display the homolog group they belong to (*a*-*e*). The super-contigs that BLAST alignments were made to are shown, including their mean coverage by sequence data during genome assembly. Alignment details are also shown, including the % of the query sequence comprising the alignment and the % of identical of sites within alignments.

FERA 1164

BLAST Queery			Homolog group	Similarity in contig:			Alignment details		
Pathotype	Gene	Length		Contig	Length	Coverage	% Query sequence	E-value	% Identity
Apple	*AMT1*	13092							
	AMT2	1254	*a*	371	596369	23	25	$9.0x10^{-26}$	69
	AMT3	1561							
	AMT4	913							
	AMT5	1223							
	AMT6	1403							
	AMT7	1797		232	459494	23	11	$1.4x10^{-12}$	69
	AMT8	2829							
	AMT9	1411							
	AMT10	2703							
	AMT11	1008		861	1931	22	6	$4.5x10^{-22}$	100
	AMT12	1248							
	AMT13	497							
	AMT14	1105							
	AMT15	555		266	10964	46	57	$4.7x10^{-44}$	70
	AMT16	1183		456	393319	23	100	0	89
	AMTR1	2461		110	64478	23	97	0	70
Pear	*AKT1*	2028	*b*						
	AKT2	849	*c*						
	AKT3	1061	*d*						
	AKTR	1335	*e*						
Strawberry	*AFT1-1*	2027	*b*						
	AFT3-1	1074	*d*						
	AFT3-2	1075	*d*						
	AFT9-1	9073							
	AFT10-1	1843		948	10583	25	8	$1.2x10^{-51}$	77
	AFT11-1	1746							
	AFT12-1	894							
	AFTS1	1252	*a*	223	368544	23	7	$7.9x10^{-14}$	79
	AFTR-1	1338	*e*						
	AFTR-2	1335	*e*						
Tangerine	*ACTT1*	785	*b*						
	ACTT2	849	*c*						
	ACTT3	1061	*d*						
	ACTTR	1308	*e*						
	ACTT5	1883							
	ACTT6	897							
Rough lemon	*ACRTS1*	1394		221	54503	23	80	$78.2x10^{-46}$	65
	ACRTS2	7958							
Tomato	*ALT1*	668							

Supplementary table 8.1*h* Results from BLASTn searches of 40 toxin synthesis genes against the genome of *FERA 1166*: Toxin genes are ordered by their associated pathotype and display the homolog group they belong to (*a*-*e*). The super-contigs that BLAST alignments were made to are shown, including their mean coverage by sequence data during genome assembly. Alignment details are also shown, including the % of the query sequence comprising the alignment and the % of identical of sites within alignments.

FERA 1166

BLAST Queery			Homolog group	Similarity in contig:			Alignment details		
Pathotype	Gene	Length		Contig	Length	Coverage	% Query sequence	E-value	% Identity
Apple	*AMT1*	13092		282	16977	53	100	0	99
	AMT2	1254	*a*	599	942641	19	100	0	100
	AMT3	1561		1588	222143	21	100	0	99
	AMT4	913		599	942641	19	100	0	97
	AMT5	1223		599	942641	19	100	0	99
	AMT6	1403		599	942641	19	100	0	97
	AMT7	1797		599	942641	19	100	0	98
	AMT8	2829		599	942641	19	100	0	99
	AMT9	1411		599	972641	19	88	0	97
	AMT10	2703		1588	222143	21	100	0	99
	AMT11	1008		1712	15118	34	95	$1.6x10^{-135}$	72
	AMT12	1248		1712	15118	34	100	0	97
	AMT13	497		1293	561743	19	100	0	100
	AMT14	1105		1293	561743	19	100	0	99
	AMT15	555		1228	57868	31	57	$4.8x10^{-44}$	70
	AMT16	1183		1293	561743	19	100	0	99
	AMTR1	2461		1588	222143	21	100	0	95
Pear	*AKT1*	2028	*b*						
	AKT2	849	*c*						
	AKT3	1061	*d*						
	AKTR	1335	*e*						
Strawberry	*AFT1-1*	2027	*b*						
	AFT3-1	1074	*d*						
	AFT3-2	1075	*d*						
	AFT9-1	9073							
	AFT10-1	1843		2645	1735204	18	8	$1.2x10^{-51}$	91
	AFT11-1	1746							
	AFT12-1	894							
	AFTS1	1252	*a*	599	942641	19	96	0	77
	AFTR-1	1338	*e*						
	AFTR-2	1335	*e*						
Tangerine	*ACTT1*	785	*b*						
	ACTT2	849	*c*						
	ACTT3	1061	*d*						
	ACTTR	1308	*e*						
	ACTT5	1883							
	ACTT6	897							
Rough lemon	*ACRTS1*	1394		160	618348	18	80	$1.6x10^{-48}$	65
	ACRTS2	7958							
Tomato	*ALT1*	668							

Supplementary table 8.1*i* Results from BLASTn searches of 40 toxin synthesis genes against the genome of *FERA 1177*: Toxin genes are ordered by their associated pathotype and display the homolog group they belong to (*a*-*e*). The super-contigs that BLAST alignments were made to are shown, including their mean coverage by sequence data during genome assembly. Alignment details are also shown, including the % of the query sequence comprising the alignment and the % of identical of sites within alignments.

FERA 1177

BLAST Queery			Homolog group	Similarity in contig:			Alignment details		
Pathotype	Gene	Length		Contig	Length	Coverage	% Query sequence	E-value	% Identity
Apple	*AMT1*	13092		36	4561	357	35	0	100
	AMT2	1254	*a*	471	2327	531	100	0	100
	AMT3	1561		154	1479	481	25	0	99
	AMT4	913		193	2528	602	100	0	97
	AMT5	1223		471	2327	531	19	$5.5x10^{-117}$	100
	AMT6	1403		460	4135	564	100	0	97
	AMT7	1797		460	4135	564	100	0	98
	AMT8	2829		150	2920	544	85	0	99
	AMT9	1411							
	AMT10	2703		176	14244	162	100	0	99
	AMT11	1008		275	8621	175	95	$1.6x10^{-135}$	72
	AMT12	1248		275	8621	175	100	0	97
	AMT13	497		358	11116	164	100	0	100
	AMT14	1105		358	11116	164	100	0	99
	AMT15	555		1239	1761	141	57	$4.8x10^{-44}$	70
	AMT16	1183		358	11116	164	100	0	99
	AMTR1	2461		176	14244	162	100	0	95
Pear	*AKT1*	2028	*b*						
	AKT2	849	*c*						
	AKT3	1061	*d*						
	AKTR	1335	*e*						
Strawberry	*AFT1-1*	2027	*b*						
	AFT3-1	1074	*d*						
	AFT3-2	1075	*d*						
	AFT9-1	9073							
	AFT10-1	1843		3708	10584	55	8	$1.2x10^{-51}$	91
	AFT11-1	1746							
	AFT12-1	894							
	AFTS1	1252	*a*	471	2327	531	96	0	77
	AFTR-1	1338	*e*						
	AFTR-2	1335	*e*						
Tangerine	*ACTT1*	785	*b*						
	ACTT2	849	*c*						
	ACTT3	1061	*d*						
	ACTTR	1308	*e*						
	ACTT5	1883							
	ACTT6	897							
Rough lemon	*ACRTS1*	1394		5868	281189	46	80	$1.6x10^{-48}$	65
	ACRTS2	7958							
Tomato	*ALT1*	668							

Supplementary table 8.1*j* Results from BLASTn searches of 40 toxin synthesis genes against the genome of *FERA 24350*: Toxin genes are ordered by their associated pathotype and display the homolog group they belong to (*a*-*e*). The super-contigs that BLAST alignments were made to are shown, including their mean coverage by sequence data during genome assembly. Alignment details are also shown, including the % of the query sequence comprising the alignment and the % of identical of sites within alignments.

FERA 24350

BLAST Queery			Homolog group	Similarity in contig:			Alignment details		
Pathotype	Gene	Length		Contig	Length	Coverage	% Query sequence	E-value	% Identity
Apple	*AMT1*	13092							
	AMT2	1254	*a*	392	570940	28	25	$8.6x10^{-26}$	69
	AMT3	1561							
	AMT4	913							
	AMT5	1223							
	AMT6	1403							
	AMT7	1797		26	1146724	28	11	$1.1x10^{-13}$	69
	AMT8	2829							
	AMT9	1411							
	AMT10	2703							
	AMT11	1008		115	2863	16	6	$1.5x10^{-21}$	98
	AMT12	1248							
	AMT13	497							
	AMT14	1105							
	AMT15	555		1337	447634	28	50	$1.4x10^{-31}$	71
	AMT16	1183		109	159610	28	100	0	89
	AMTR1	2461		130	66072	29	97	0	70
Pear	*AKT1*	2028	*b*						
	AKT2	849	*c*						
	AKT3	1061	*d*						
	AKTR	1335	*e*						
Strawberry	*AFT1-1*	2027	*b*						
	AFT3-1	1074	*d*						
	AFT3-2	1075	*d*						
	AFT9-1	9073							
	AFT10-1	1843							
	AFT11-1	1746							
	AFT12-1	894							
	AFTS1	1252	*a*	1503	476594	28	7	$7.5x10^{-14}$	79
	AFTR-1	1338	*e*						
	AFTR-2	1335	*e*						
Tangerine	*ACTT1*	785	*b*						
	ACTT2	849	*c*						
	ACTT3	1061	*d*						
	ACTTR	1308	*e*						
	ACTT5	1883							
	ACTT6	897							
Rough lemon	*ACRTS1*	1394		140	1141049	29	80	$7.8x10^{-46}$	65
	ACRTS2	7958							
Tomato	*ALT1*	668							

Supplementary table 8.1*k* Results from BLASTn searches of 40 toxin synthesis genes against the genome of *RGR 97.0013*: Toxin genes are ordered by their associated pathotype and display the homolog group they belong to (*a*-*e*). The super-contigs that BLAST alignments were made to are shown, including their mean coverage by sequence data during genome assembly. Alignment details are also shown, including the % of the query sequence comprising the alignment and the % of identical of sites within alignments.

FERA 97.0013

BLAST Queery			Homolog group	Similarity in contig:			Alignment details		
Pathotype	Gene	Length		Contig	Length	Coverage	% Query sequence	E-value	% Identity
Apple	*AMT1*	13092							
	AMT2	1254	*a*	392	570940	28	25	$8.6x10^{-26}$	69
	AMT3	1561							
	AMT4	913							
	AMT5	1223							
	AMT6	1403							
	AMT7	1797		26	114724	28	11	$1.1x10^{-13}$	69
	AMT8	2829							
	AMT9	1411							
	AMT10	2703							
	AMT11	1008		1155	2863	16	6	$1.5x10^{-21}$	98
	AMT12	1248							
	AMT13	497							
	AMT14	1105							
	AMT15	555		1337	447634	28	50	$1.4x10^{-31}$	71
	AMT16	1183		109	159610	29	100	0	89
	AMTR1	2461		130	66072	29	97	0	70
Pear	*AKT1*	2028	*b*						
	AKT2	849	*c*						
	AKT3	1061	*d*						
	AKTR	1335	*e*						
Strawberry	*AFT1-1*	2027	*b*						
	AFT3-1	1074	*d*						
	AFT3-2	1075	*d*						
	AFT9-1	9073							
	AFT10-1	1843							
	AFT11-1	1746							
	AFT12-1	894							
	AFTS1	1252	*a*	127	978690	29	7	$7.6x10^{-14}$	79
	AFTR-1	1338	*e*						
	AFTR-2	1335	*e*						
Tangerine	*ACTT1*	785	*b*						
	ACTT2	849	*c*						
	ACTT3	1061	*d*						
	ACTTR	1308	*e*						
	ACTT5	1883							
	ACTT6	897							
Rough lemon	*ACRTS1*	1394		397	1263283	29	80	$1.6x10^{-54}$	65
	ACRTS2	7958							
Tomato	*ALT1*	668							

Supplementary table 8.1*l* Results from BLASTn searches of 40 toxin synthesis genes against the genome of *RGR 97.0016*: Toxin genes are ordered by their associated pathotype and display the homolog group they belong to (*a*-*e*). The super-contigs that BLAST alignments were made to are shown, including their mean coverage by sequence data during genome assembly. Alignment details are also shown, including the % of the query sequence comprising the alignment and the % of identical of sites within alignments.

FERA 97.0016

BLAST Queery			Homolog group	Similarity in contig:			Alignment details		
Pathotype	Gene	Length		Contig	Length	Coverage	% Query sequence	E-value	% Identity
Apple	*AMT1*	13092							
	AMT2	1254	*a*	199	152710	22	25	$2.04x10^{-27}$	70
	AMT3	1561							
	AMT4	913							
	AMT5	1223							
	AMT6	1403							
	AMT7	1797		646	746184	22	11	$1.1x10^{-13}$	69
	AMT8	2829							
	AMT9	1411							
	AMT10	2703							
	AMT11	1008		916	119322	22	5	$1.9x10^{-20}$	100
	AMT12	1248							
	AMT13	497							
	AMT14	1105							
	AMT15	555							
	AMT16	1183		194	709120	22	100	0	89
	AMTR1	2461		394	178184	21	97	0	69
Pear	*AKT1*	2028	*b*						
	AKT2	849	*c*						
	AKT3	1061	*d*						
	AKTR	1335	*e*						
Strawberry	*AFT1-1*	2027	*b*						
	AFT3-1	1074	*d*						
	AFT3-2	1075	*d*						
	AFT9-1	9073							
	AFT10-1	1843							
	AFT11-1	1746							
	AFT12-1	894							
	AFTS1	1252	*a*	372	92	22	7	$7.6x10^{-14}$	79
	AFTR-1	1338	*e*						
	AFTR-2	1335	*e*						
Tangerine	*ACTT1*	785	*b*						
	ACTT2	849	*c*						
	ACTT3	1061	*d*						
	ACTTR	1308	*e*						
	ACTT5	1883							
	ACTT6	897							
Rough lemon	*ACRTS1*	1394		854	287896	22	80	$1.6x10^{-54}$	65
	ACRTS2	7958							
Tomato	*ALT1*	668							

Supplementary table 8.2*a* 86 *Saccharomyces cerevisiae* genes involved in meiosis and gene IDs of their homologs in sexual and non sexual Dothideomycetes (expanded): As identified by reciprocal BLAST searches in genome sequence of: three strains of *Alternaria alternata* (putatively asexual), *Pyrenophora tritici-repentis* (***Py. tr***: sexual), *Cochliobolus heterostrophus* (***Co. h***: sexual), *Phaeosphaeria nodorum* (***Ph. n***: sexual) and *Alternaria brassicicola* (***Al. b***: putatively asexual). The 29 core meiotic genes for Eukaryotes (CMG) and nine meiosis-specific genes (MSG) are marked.

Meiotic gene:				Sexual species:			*Alternaria alternata:*				CMG	MSG
Process	**Name**	**Description**	**Gene ID**	***Py. tr***	***Co. h***	***Ph. n***	***FERA 675***	***FERA 1166***	***FERA 650***	***Al. b***		
Generation of double stranded breaks	*SPO11*	Meiosis-specific protein that initiates meiotic recombination by catalyzing the formation of double-strand breaks in DNA via a transesterification reaction; required for homologous chromosome pairing and synaptonemal complex formation	*YHL022C SPAC17A5.11*	*PTRG_09549*			*6413*	*7789*	*1374*	*AB01004*	X	X
	REC107 /MEI2	Protein involved in early stages of meiotic recombination; involved in coordination between the initiation of recombination and the first division of meiosis; part of a complex (Rec107p-Mei4p-Rec114p) required for DS break formation	*YJR021C*									
	MEI4	Meiosis-specific protein involved in recombination; required for chromosome synapsis; required for production of viable spores	*YER044C*	*PRT_11205*	*32878*	*6690*	*6079*	*752*	*3918*			
	REC102	Protein involved in early stages of meiotic recombination; required for chromosome synapsis; forms a complex with Rec104p and Spo11p necessary during the initiation of recombination	*YLR329W*									
	REC104	Protein involved in early stages of meiotic recombination; required for meiotic crossing over; forms a complex with Rec102p and Spo11p necessary during the initiation of recombination	*YHR157W*									
	REC114	Protein involved in early stages of meiotic recombination; possibly involved in the coordination of recombination and meiotic division; mutations lead to premature initiation of the first meiotic division	*YMR133W SPCC1753.03 c*									
	SKI8	Protein involved in exosome mediated 3' to 5' mRNA degradation and translation inhibition of non-poly(A) mRNAs as well as double-strand break formation during meiotic recombination; required for repressing propagation of dsRNA viruses	*YGL213C*	*PTRG_11378*	*25636*	*11213*	*1515*	*5412*	*2967*	*AB03718*		
	MER1	Protein with RNA-binding motifs required for meiosis-specific mRNA splicing; required for chromosome pairing and meiotic recombination	*YNL210W*									
	HFM1 /MER3	Meiosis specific DNA helicase involved in the conversion of double-stranded breaks to later recombination intermediates and in crossover control; catalyzes the unwinding of Holliday junctions; has ssDNA and dsDNA stimulated ATPase activity	*YGL251C*	*PRT_01390*	*68304*	*12310*	*646*	*3402*	*2455*		X	X
	NAM8 /MRE2	RNA binding protein, component of the U1 snRNP protein; mutants are defective in meiotic recombination and in formation of viable spores, involved in the formation of DSBs through meiosis-specific splicing of MER2 pre-mRNA	*YHR086W*	*PRT_04758*	*95538*	*4597*	*2900*	*2549*	*3127*	*AB03394*		

Supplementary table 8.2*b* 86 *Saccharomyces cerevisiae* genes involved in meiosis and gene IDs of their homologs in sexual and non sexual Dothideomycetes (expanded): As identified by reciprocal BLAST searches in genome sequence of: three strains of *Alternaria alternata* (putatively asexual), *Pyrenophora tritici-repentis* (***Py. tr***: sexual), *Cochliobolus heterostrophus* (***Co. h***: sexual), *Phaeosphaeria nodorum* (***Ph. n***: sexual) and *Alternaria brassicicola* (***Al. b***: putatively asexual). The 29 core meiotic genes for Eukaryotes (CMG) and nine meiosis-specific genes (MSG) are marked.

Meiotic gene:				Sexual species:			*Alternaria alternata:*				CMG	MSG
Process	**Name**	**Description**	**Gene ID**	***Py. tr***	***Co. h***	***Ph. n***	***FERA 675***	***FERA 1166***	***FERA 650***	***Al. b***		
Removal of Spo11	*MRE11*	Subunit of a complex with Rad50p and Xrs2p (RMX complex) that functions in repair of DNA double-strand breaks and in telomere stability, exhibits nuclease activity that appears to be required for RMX function; widely conserved	*YMR224C*	*PRT_00585*	*117005*	*8249*	*7822*	*1276*	*7236*	*AB03216*	X	
	RAD50	Subunit of MRX complex, with Mre11p and Xrs2p, involved in processing double-strand DNA breaks in vegetative cells, initiation of meiotic DSBs, telomere maintenance, and nonhomologous end joining	*YNL250W*	*PTRG_04512*		*7691*	*5008*	*6401*	*7020*	*AB07722*	X	
	XRS2 /NBS1	Protein required for DNA repair; component of the Mre11 complex, which is involved in double strand breaks, meiotic recombination, telomere maintenance, and checkpoint signaling	*YDR369C SPBC6B1.09c*	*PTRG_07427*						*ABO6739*		
	SAE2 /COM1	Protein with a role in accurate meiotic and mitotic double-strand break repair; phosphorylated in response to DNA damage and required for normal resistance to DNA-damaging agents	*YGL175C*	*PTRG_3670*	*107945*		*12703*	*11712*	*6422*	*AB06233*		

Supplementary table 8.2*c* 86 *Saccharomyces cerevisiae* genes involved in meiosis and gene IDs of their homologs in sexual and non sexual Dothideomycetes (expanded): As identified by reciprocal BLAST searches in genome sequence of: three strains of *Alternaria alternata* (putatively asexual), *Pyrenophora tritici-repentis* (***Py. tr***: sexual), *Cochliobolus heterostrophus* (***Co. h***: sexual), *Phaeosphaeria nodorum* (***Ph. n***: sexual) and *Alternaria brassicicola* (***Al. b***: putatively asexual). The 29 core meiotic genes for Eukaryotes (CMG) and nine meiosis-specific genes (MSG) are marked.

Meiotic gene:				Sexual species:			*Alternaria alternata:*				CMG	MSG
Process	**Name**	**Description**	**Gene ID**	***Py. tr***	***Co. h***	***Ph. n***	***FERA 675***	***FERA 1166***	***FERA 650***	***Al. b***		
Strand invasion	*RAD51*	Strand exchange protein, forms a helical filament with DNA that searches for homology; involved in the recombinational repair of double-strand breaks in DNA during vegetative growth and meiosis; homolog of Dmc1p and bacterial RecA protein	*YER095W NCU02741.1*	*PTRG_11408*	*27223*	*8230*	*12540*	*8547*	*9598*	*AB07310*	X	
	DMC1	Meiosis-specific protein required for repair of double-strand breaks and pairing between homologous chromosomes; homolog of Rad51p and the bacterial RecA protein	*YER179W SPAC8E11.03c*	*PTRG_04810*		*4647*	*12010*	*2703*	*10324*	*AB03512*	X	X
	RAD52	Protein that stimulates strand exchange by facilitating Rad51p binding to single-stranded DNA; anneals complementary single-stranded DNA; involved in the repair of double-strand breaks in DNA during vegetative growth and meiosis	*YML032C NCU04275.1*	*PTRG_03837*	*30327*	*11210*	*1514*	*5413*	*2968*	*AB03717*	X	
	RAD54	DNA-dependent ATPase, stimulates strand exchange by modifying the topology of double-stranded DNA; involved in the recombinational repair of double-strand breaks in DNA during vegetative growth and meiosis; member of the SWI/SNF family	*YGL163C NCU02348.1*	*PTRG_11570*	*118646*	*9338*	*10426*	*10707*	*6371*	*AB09353*		
	RDH54	DNA-dependent ATPase, stimulates strand exchange by modifying the topology of double-stranded DNA; involved in recombinational repair of DNA double-strand breaks during mitosis and meiosis; proposed to be involved in crossover interference	*YBR073W*	*PTRG_11570*	*29888*	*7159*	*7981*	*11414*	*4361*	*AB01447*		
	RFA1	Subunit of heterotrimeric Replication Protein A (RPA), which is a highly conserved single-stranded DNA binding protein involved in DNA replication, repair, and recombination	*YAR007C NCU03606.1*		*91325*	*9020*	*160*	*11648*	*5102*	*AB08426*		
	RFA2	Subunit of heterotrimeric Replication Protein A (RPA), which is a highly conserved single-stranded DNA binding protein involved in DNA replication, repair, and recombination	*YNL312W NCU07717.1*	*PTRG_00909*	*84841*	*1218*	*7323*	*1512*	*12324*	*AB07528*		
	RFA3	Subunit of heterotrimeric Replication Protein A (RPA), which is a highly conserved single-stranded DNA binding protein involved in DNA replication, repair, and recombination	*YJL173C*									
	SAE3	Meiosis specific protein involved in DMC1-dependent meiotic recombination, forms heterodimer with Mei5p; proposed to be an assembly factor for Dmc1p	*YHR079C-A*	*PTRG_01044*		*2839*	*11661*	*12389*	*11022*	*AB09179*		
	RAD55	Protein that stimulates strand exchange by stabilizing the binding of Rad51p to single-stranded DNA; involved in the recombinational repair of double-strand breaks in DNA during vegetative growth and meiosis; forms heterodimer with Rad57p	*YDR076W*	*PTRG1184*	*32862*	*6684*	*6053*	*982*	*1593*	*AB01818*		

Supplementary table 8.2*d* 86 *Saccharomyces cerevisiae* genes involved in meiosis and gene IDs of their homologs in sexual and non sexual Dothideomycetes (expanded): As identified by reciprocal BLAST searches in genome sequence of: three strains of *Alternaria alternata* (putatively asexual), *Pyrenophora tritici-repentis* (***Py. tr***: sexual), *Cochliobolus heterostrophus* (***Co. h***: sexual), *Phaeosphaeria nodorum* (***Ph. n***: sexual) and *Alternaria brassicicola* (***Al. b***: putatively asexual). The 29 core meiotic genes for Eukaryotes (CMG) and nine meiosis-specific genes (MSG) are marked.

Meiotic gene:				Sexual species:			*Alternaria alternata:*				CMG	MSG
Process	**Name**	**Description**	**Gene ID**	***Py. tr***	***Co. h***	***Ph. n***	***FERA 675***	***FERA 1166***	***FERA 650***	***Al. b***		
DNA damage checkpoint	*PCH2*	Nucleolar component of the pachytene checkpoint, which prevents chromosome segregation when recombination and chromosome synapsis are defective; also represses meiotic interhomolog recombination in the rDNA	*YBR186W*									
	MEC1	Genome integrity checkpoint protein and PI kinase superfamily member; signal transducer required for cell cycle arrest and transcriptional responses prompted by damaged or unreplicated DNA; monitors and participates in meiotic recombination	*YBR136W*	*PTRG_08160*	*28959*	*2187*	*8000*	*11434*	*4381*	*AB01461*		
	RAD17	Checkpoint protein, involved in the activation of the DNA damage and meiotic pachytene checkpoints; with Mec3p and Ddc1p, forms a clamp that is loaded onto partial duplex DNA; homolog of human and S. pombe Rad1 and U. maydis Rec1 proteins	*YOR368W*	*PTRG_04586*	*118811*	*4583*		*9796*	*9264*	*ABO3039*		
	RAD24	Checkpoint protein, involved in the activation of the DNA damage and meiotic pachytene checkpoints; subunit of a clamp loader that loads Rad17p-Mec3p-Ddc1p onto DNA; homolog of human and S. pombe Rad17 protein	*YER173W*	*PTRG_07862*	*28078*	*10570*	*1415*	*9143*	*28*	*AB02822*		
	DDC1	DNA damage checkpoint protein, part of a PCNA-like complex required for DNA damage response, required for pachytene checkpoint to inhibit cell cycle in response to unrepaired recombination intermediates; potential Cdc28p substrate	*YPL194W*									

Supplementary table 8.2*e* 86 *Saccharomyces cerevisiae* genes involved in meiosis and gene IDs of their homologs in sexual and non sexual Dothideomycetes (expanded): As identified by reciprocal BLAST searches in genome sequence of: three strains of *Alternaria alternata* (putatively asexual), *Pyrenophora tritici-repentis* (***Py. tr***: sexual), *Cochliobolus heterostrophus* (***Co. h***: sexual), *Phaeosphaeria nodorum* (***Ph. n***: sexual) and *Alternaria brassicicola* (***Al. b***: putatively asexual). The 29 core meiotic genes for Eukaryotes (CMG) and nine meiosis-specific genes (MSG) are marked.

Meiotic gene:				Sexual species:			*Alternaria alternata:*				CMG	MSG
Process	**Name**	**Description**	**Gene ID**	***Py. tr***	***Co. h***	***Ph. n***	***FERA 675***	***FERA 1166***	***FERA 650***	***Al. b***		
Regulation of crossover frequency	*MLH1*	Protein required for mismatch repair in mitosis and meiosis as well as crossing over during meiosis; forms a complex with Pms1p and Msh2p-Msh3p during mismatch repair; human homolog is associated with hereditary non-polyposis colon cancer	*YMR167W*	*PTRG_09008*	*99634*	*2500*	*3784*	*3771*	*6573*	*AB00578*	X	
	MLH3	Protein involved in DNA mismatch repair and crossing-over during meiotic recombination; forms a complex with Mlh1p; mammalian homolog is implicated mammalian microsatellite instability	*YPL164C*	*PTRG_04295*	*106087*	*11018*	*8339*	*12200*	*3029*	*AB03674*	X	
	MSH4	Protein involved in meiotic recombination, required for normal levels of crossing over, colocalizes with Zip2p to discrete foci on meiotic chromosomes, has homology to bacterial MutS protein	*YFL003C*	*PTRG_03000*		*10193*	*8092*	*5697*	*5641*	*AB08026*	X	X
	MSH5	Protein of the MutS family, forms a dimer with Msh4p that facilitates crossovers between homologs during meiosis; msh5-Y823H mutation confers tolerance to DNA alkylating agents; homologs present in C. elegans and humans	*YDL154W*	*PTRG_00548*	*34576*	*1172*	*7788*	*4184*	*7200*	*AB07744*	X	X
	SGS1	Nucleolar DNA helicase of the RecQ family involved in maintenance of genome integrity, regulates chromosome synapsis and meiotic crossing over; has similarity to human BLM and WRN helicases implicated in Bloom and Werner syndromes	*YMR190C*	*PTRG_06397*	*33244*	*5678*	*6109*	*10265*	*3886*	*AB01867*		
	MEI5	Meiosis specific protein involved in DMC1-dependent meiotic recombination, forms heterodimer with Sae3p; proposed to be an assembly factor for Dmc1p	*YPL121C*	*PTRG_04633*	*32063*	*7660*				*AB03519*		
	MUM2	Cytoplasmic protein essential for meiotic DNA replication and sporulation; interacts with Orc2p, which is a component of the origin recognition complex	*YBR057C*									
	NDJ1	Meiosis-specific telomere protein, required for bouquet formation, effective homolog pairing, ordered cross-over distribution (interference), sister chromatid cohesion at meiotic telomeres, and segregation of small chromosomes	*YOL104C*									
	RAD1	Single-stranded DNA endonuclease (with Rad10p), cleaves single-stranded DNA during nucleotide excision repair and double-strand break repair; subunit of Nucleotide Excision Repair Factor 1 (NEF1); homolog of human XPF protein, meiotic chromosome segregation; reciprocal meiotic recombination; resolution of meiotic joint molecules as recombinants.	*YPL022W*	*PTRG_05712*	*117597*	*1485*	*10304*	*10315*	*6961*	*AB07181*	X	
	RAD2	Single-stranded DNA endonuclease, cleaves single-stranded DNA during nucleotide excision repair to excise damaged DNA; subunit of Nucleotide Excision Repair Factor 3 (NEF3); homolog of human XPG protein	*YGR258C*	*PTRG_07140*	*101055*	*732*	*1889*	*492*	*5485*	*AB08822*		

Supplementary table 8.2*f* 86 *Saccharomyces cerevisiae* genes involved in meiosis and gene IDs of their homologs in sexual and non sexual Dothideomycetes (expanded): As identified by reciprocal BLAST searches in genome sequence of: three strains of *Alternaria alternata* (putatively asexual), *Pyrenophora tritici-repentis* (***Py. tr***: sexual), *Cochliobolus heterostrophus* (***Co. h***: sexual), *Phaeosphaeria nodorum* (***Ph. n***: sexual) and *Alternaria brassicicola* (***Al. b***: putatively asexual). The 29 core meiotic genes for Eukaryotes (CMG) and nine meiosis-specific genes (MSG) are marked.

Meiotic gene:				Sexual species:			*Alternaria alternata:*				CMG	MSG
Process	**Name**	**Description**	**Gene ID**	***Py. tr***	***Co. h***	***Ph. n***	***FERA 675***	***FERA 1166***	***FERA 650***	***Al. b***		
Formation of synaptonemal complex	*HOP1*	Meiosis-specific DNA binding protein that displays Red1p dependent localization to the unsynapsed axial-lateral elements of the synaptonemal complex; required for homologous chromosome synapsis and chiasma formation	*YIL072W*								X	X
	HOP2	Meiosis-specific protein that localizes to chromosomes, preventing synapsis between nonhomologous chromosomes and ensuring synapsis between homologs; complexes with Mnd1p to promote homolog pairing and meiotic double-strand break repair	*YGL033W, SPAC222.15*	*PGRT_06257*	*76108*	*7934*	*4248*	*2082*			X	X
	MND1	Protein required for recombination and meiotic nuclear division; forms a complex with Hop2p, which is involved in chromosome pairing and repair of meiotic double-strand breaks	*YGL183C SPAC13A11.03*	*PTRG_08720*	*115062*		*8132*		*5680*	*AB08058*	X	X
	ZIP1	Transverse filament protein of the synaptonemal complex; required for normal levels of meiotic recombination and pairing between homologous chromosome during meiosis; potential Cdc28p substrate	*YDR285W NCU00658*									
	ZIP2	Meiosis-specific protein involved in normal synaptonemal complex formation and pairing between homologous chromosomes during meiosis	*YGL249W*									
	ZIP3	SUMO E3 ligase; required for synaptonemal complex formation; localizes to synapsis initiation sites on meiotic chromosomes; potential Cdc28p substrate	*YLR394W*									
	Zip4 /Spo22	Meiosis-specific protein essential for chromosome synapsis, involved in completion of nuclear divisions during meiosis; induced early in meiosis	*YIL073C*		*66733*							

Supplementary table 8.2*g* 86 *Saccharomyces cerevisiae* genes involved in meiosis and gene IDs of their homologs in sexual and non sexual Dothideomycetes (expanded): As identified by reciprocal BLAST searches in genome sequence of: three strains of *Alternaria alternata* (putatively asexual), *Pyrenophora tritici-repentis* (***Py. tr***: sexual), *Cochliobolus heterostrophus* (***Co. h***: sexual), *Phaeosphaeria nodorum* (***Ph. n***: sexual) and *Alternaria brassicicola* (***Al. b***: putatively asexual). The 29 core meiotic genes for Eukaryotes (CMG) and nine meiosis-specific genes (MSG) are marked.

Meiotic gene:				Sexual species:			*Alternaria alternata:*				CMG	MSG
Process	**Name**	**Description**	**Gene ID**	***Py. tr***	***Co. h***	***Ph. n***	***FERA 675***	***FERA 1166***	***FERA 650***	***Al. b***		
DNA repair	*HTA1*	Histone H2A, core histone protein required for chromatin assembly and chromosome function; one of two nearly identical subtypes (see also HTA2); DNA damage-dependent phosphorylation by Mec1p facilitates DNA repair; acetylated by Nat4p	*YDR225W SPAC19G12.06c*				*4291*	*2039*	*6685*			
	HTA2	Histone H2A, core histone protein required for chromatin assembly and chromosome function; one of two nearly identical (see also HTA1) subtypes; DNA damage-dependent phosphorylation by Mec1p facilitates DNA repair; acetylated by Nat4p	*YBL003C*	*PTRG_3280*	*106518*	*8011*				*AB02240*		
	RED1	Protein component of the axial elements of the synaptonemal complex, involved in chromosome segregation during the first meiotic division; interacts with Hop1p; required for wild-type levels of Mek1p kinase activity	*YLR263W*									
	SMC5	Structural maintenance of chromosomes (SMC) protein; essential subunit of the Mms21-Smc5-Smc6 complex; required for growth and DNA repair; S. pombe homolog forms a heterodimer with S. pombe Rad18p that is involved in DNA repair	*YOL034W*	*PTRG_05673*	*67121*	*1468*	*10318*	*10302*	*6946*	*AB07165*	**X**	
	SMC6	Protein involved in structural maintenance of chromosomes; essential subunit of Mms21-Smc5-Smc6 complex; required for growth, DNA repair, interchromosomal and sister chromatid recombination; homologous to S. pombe rad18	*YLR383W*	*PTRG_06260*	*113791*	*7950*	*4257*	*2072*	*6720*	*AB02217*	**X**	
	EXO1	5'-3' exonuclease and flap-endonuclease involved in recombination, double-strand break repair and DNA mismatch repair; member of the Rad2p nuclease family, with conserved N and I nuclease domains	*YOR033C SPBC29A10.05*	*PTRG_06451*	*106358*	*7985*	*8928*	*1217*	*4291*	*AB01618*		
	HRR25	Protein kinase involved in regulating diverse events including vesicular trafficking, DNA repair, and chromosome segregation; binds the CTD of RNA pol II; homolog of mammalian casein kinase 1delta (CK1delta)	*YPL204W SPBC3H7.15*	*PTRG_07154*	*116470*	*261*	*2045*	*659*	*9879*			
	RAD23	Protein with ubiquitin-like N terminus, recognizes and binds damaged DNA (with Rad4p) during nucleotide excision repair; regulates Rad4p levels, subunit of Nuclear Excision Repair Factor 2 (NEF2); homolog of human HR23A and HR23B proteins	*YEL037C SPBC2D10.12*	*PTRG_03535*	*89218*	*4837*	*12464*	*7608*	*9111*	*AB05871*		

Supplementary table 8.2*h* 86 *Saccharomyces cerevisiae* genes involved in meiosis and gene IDs of their homologs in sexual and non sexual Dothideomycetes (expanded): As identified by reciprocal BLAST searches in genome sequence of: three strains of *Alternaria alternata* (putatively asexual), *Pyrenophora tritici-repentis* (***Py. tr***: sexual), *Cochliobolus heterostrophus* (***Co. h***: sexual), *Phaeosphaeria nodorum* (***Ph. n***: sexual) and *Alternaria brassicicola* (***Al. b***: putatively asexual). The 29 core meiotic genes for Eukaryotes (CMG) and nine meiosis-specific genes (MSG) are marked.

Meiotic gene:				Sexual species:			*Alternaria alternata:*				CMG	MSG
Process	**Name**	**Description**	**Gene ID**	***Py. tr***	***Co. h***	***Ph. n***	***FERA 675***	***FERA 1166***	***FERA 650***	***Al. b***		
Mismatch repair	*MSH2*	Protein that forms heterodimers with Msh3p and Msh6p that bind to DNA mismatches to initiate the mismatch repair process; contains a Walker ATP-binding motif required for repair activity; Msh2p-Msh6p binds to and hydrolyzes ATP	*YOL090W*	*PTRG_11532*	*97160*	*3061*	*8292*	*5939*	*9712*	*AB01346*	X	
	MSH3	Mismatch repair protein, forms dimers with Msh2p that mediate repair of insertion or deletion mutations and removal of nonhomologous DNA ends, contains a PCNA (Pol30p) binding motif required for genome stability	*YCR092C*	*PTRG_02865*	*118412*	*10028*	*12415*	*11842*	*7365*	*AB07868*		
	MSH6	Protein required for mismatch repair in mitosis and meiosis, forms a complex with Msh2p to repair both single-base & insertion-deletion mispairs; potentially phosphorylated by Cdc28p	*YDR097C*	*PTRG_06916*	*28555*	*665*	*1776*	*11005*	*6312*	*AB06674*	X	
	MLH2	ATP-binding protein required for mismatch repair in mitosis and meiosis; functions as a heterodimer with Mlh1p, binds double- and single-stranded DNA via its N-terminal domain, homologous to E. coli MutL	*YLR035C*								X	
	PMS1	ATP-binding protein required for mismatch repair in mitosis and meiosis; functions as a heterodimer with Mlh1p, binds double- and single-stranded DNA via its N-terminal domain, homologous to E. coli MutL	*YNL082W*	*PTRG_04920*	*119479*	*11888*	*5383*	*4973*	*9458*	*AB05713*	X	

Supplementary table 8.2*i* 86 *Saccharomyces cerevisiae* genes involved in meiosis and gene IDs of their homologs in sexual and non sexual Dothideomycetes (expanded): As identified by reciprocal BLAST searches in genome sequence of: three strains of *Alternaria alternata* (putatively asexual), *Pyrenophora tritici-repentis* (***Py. tr***: sexual), *Cochliobolus heterostrophus* (***Co. h***: sexual), *Phaeosphaeria nodorum* (***Ph. n***: sexual) and *Alternaria brassicicola* (***Al. b***: putatively asexual). The 29 core meiotic genes for Eukaryotes (CMG) and nine meiosis-specific genes (MSG) are marked.

Meiotic gene:				Sexual species:			*Alternaria alternata:*				CMG	MSG
Process	**Name**	**Description**	**Gene ID**	***Py. tr***	***Co. h***	***Ph. n***	***FERA 675***	***FERA 1166***	***FERA 650***	***Al. b***		
Resolution of recombination intermediates	*MMS4*	Subunit of the structure-specific Mms4p-Mus81p endonuclease that cleaves branched DNA; involved in recombination and DNA repair	*YBR098W*									
	MUS81	Helix-hairpin-helix protein, involved in DNA repair and replication fork stability; functions as an endonuclease in complex with Mms4p; interacts with Rad54p	*YDR386W*									
	SLX1	Subunit of a complex, with Slx4p, that hydrolyzes 5' branches from duplex DNA in response to stalled or converging replication forks; function overlaps with that of Sgs1p-Top3p	*YBR228W*	*PTRG_11044*	*85914*	*4329*	*3679*	*7849*	*10058*	*AB01206*		
	TOP1	Topoisomerase I, nuclear enzyme that relieves torsional strain in DNA by cleaving and re-sealing the phosphodiester backbone; relaxes both positively and negatively supercoiled DNA; functions in replication, transcription, and recombination	*YOL006C*	*PTRG_07613*	*27904*	*10838*	*2818*	*6287*	*11769*	*AB03317*		
	TOP2	Essential type II topoisomerase, relieves torsional strain in DNA by cleaving and re-sealing the phosphodiester backbone of both positively and negatively supercoiled DNA; cleaves complementary strands; localizes to axial cores in meiosis	*YNL088W*	*PTRG_02676*	*107366*	*12111*	*11352*	*3546*	*9170*	*AB04715*		
	TOP3	DNA Topoisomerase III, conserved protein that functions in a complex with Sgs1p and Rmi1p to relax single-stranded negatively-supercoiled DNA preferentially, involved in telomere stability and regulation of mitotic recombination	*YLR234W*	*PTRG_00118*	*58344*	*8055*	*4186*	*5075*	*4483*	*AB04014*		
	SLX4	Subunit of a complex, with Slx1p, that hydrolyzes 5' branches from duplex DNA in response to stalled or converging replication forks; function overlaps with that of Sgs1p-Top3p	*YLR135W*									
	SLX5	Subunit of the Slx5-Slx8 substrate-specific ubiquitin ligase complex; stimulated by prior attachment of SUMO to the substrate	*YDL013W*	*PTRG_05763*								
	SLX8	Subunit of the Slx5-Slx8 substrate-specific ubiquitin ligase complex; stimulated by prior attachment of SUMO to the substrate	*YER116C*									
Joining of non-homologous ends	*YKU70*	Subunit of the telomeric Ku complex (Yku70p-Yku80p), involved in telomere length maintenance, structure and telomere position effect; relocates to sites of double-strand cleavage to promote nonhomologous end joining during DSB repair	*YMR284W*	*PGRT_10775*	*32946*	*6240*	*9454*	*289*	*5448*	*ABO6267*		
	YKU80	Subunit of the telomeric Ku complex (Yku70p-Yku80p), involved in telomere length maintenance, structure and telomere position effect; relocates to sites of double-strand cleavage to promote nonhomologous end joining during DSB repair	*YMR106C*	*PTRG_00307*		*5411*	*7715*	*7377*	*4631*	*AB04153*		
	DNL4	DNA ligase required for nonhomologous end-joining (NHEJ), forms stable heterodimer with required cofactor Lif1p, interacts with Nej1p; involved in meiosis, not essential for vegetative growth	*YOR005C*	*PTRG_09982*	*98077*	*3843*	*3270*	*11559*	*575*	*AB00272*		
	LIF1	Protein involved in DNA double-strand break repair; physically interacts with DNA ligase 4 (Lig4p); homologous to mammalian XRCC4 protein	*YGL090W*	*PTRG_03968*						*AB06175*		

Supplementary table 8.2*j* 86 *Saccharomyces cerevisiae* genes involved in meiosis and gene IDs of their homologs in sexual and non sexual Dothideomycetes (expanded): As identified by reciprocal BLAST searches in genome sequence of: three strains of *Alternaria alternata* (putatively asexual), *Pyrenophora tritici-repentis* (***Py. tr***: sexual), *Cochliobolus heterostrophus* (***Co. h***: sexual), *Phaeosphaeria nodorum* (***Ph. n***: sexual) and *Alternaria brassicicola* (***Al. b***: putatively asexual). The 29 core meiotic genes for Eukaryotes (CMG) and nine meiosis-specific genes (MSG) are marked.

Meiotic gene:				Sexual species:			*Alternaria alternata:*				CMG	MSG
Process	Name	Description	Gene ID	*Py. tr*	*Co. h*	*Ph. n*	*FERA 675*	*FERA 1166*	*FERA 650*	*Al. b*		
Other	*MSC1*	Protein of unknown function; mutant is defective in directing meiotic recombination events to homologous chromatids; the authentic, non-tagged protein is detected in highly purified mitochondria and is phosphorylated	*YML128C*	*PTRG_08369*	*28775*	*1920*	*7425*	*903*	*7101*	*AB02609*		
	MSC7	Protein of unknown function, green fluorescent protein (GFP)-fusion protein localizes to the endoplasmic reticulum; msc7 mutants are defective in directing meiotic recombination events to homologous chromatids	*YHR039C*	*PTRG_05412*	*98545*	*1786*	*10676*	*4641*	*5311*	*AB010021*		
	MSC3	Protein of unknown function, green fluorescent protein (GFP)-fusion protein localizes to the cell periphery; msc3 mutants are defective in directing meiotic recombination events to homologous chromatids; potential Cdc28p substrate	*YLR219W*									
	MSC6	Protein of unknown function; mutant is defective in directing meiotic recombination events to homologous chromatids; the authentic, non-tagged protein is detected in highly purified mitochondria in high-throughput studies	*YOR354C*									
	SRS2	DNA helicase and DNA-dependent ATPase involved in DNA repair, needed for proper timing of commitment to meiotic recombination and transition from Meiosis I to II; blocks trinucleotide repeat expansion; affects genome stability	*YJL092W*	*PTRG_06876*	*83246*	*765*	*7077*	*389*	*9629*	*AB08725*		
	MPS3	Nuclear envelope protein required for SPB duplication and nuclear fusion; localizes to the SPB half bridge and at telomeres during meiosis; required with Ndj1p and Csm4p for meiotic bouquet formation and telomere-led rapid prophase movement	*YJL019W*									
	REC8	Meiosis-specific component of sister chromatid cohesion complex; maintains cohesion between sister chromatids during meiosis I; maintains cohesion between centromeres of sister chromatids until meiosis	*YPR007C*	*PTRG_05324*	*16758*		*5624*	*4832*	*10527*		X	X
	RAD21	Essential alpha-kleisin subunit of the cohesin complex; required for sister chromatid cohesion in mitosis and meiosis; apoptosis induces cleavage and translocation of a C-terminal fragment to mitochondria	*YDL003W*	*PTRG_11702*	*31769*	*9049*	*195*	*11609*	*5067*	*AB08415*	X	
	SMC1	Subunit of the multiprotein cohesin complex, essential protein for chromosome segregation and in double-strand DNA break repair	*YFL008W*	*PTRG_07093*		*578*	*11698*	*8929*	*8459*	*AB06593*	X	
	SMC2	Subunit of the condensin complex that forms a complex with Smc4p to form the active ATPase	*YFR031C*	*PTRG_11170*	*116271*	*5966*	*4410*	*1913*	*323*	*AB02349*	X	
	SMC3	Subunit of the multiprotein cohesin complex required for sister chromatid cohesion in mitotic cells	*YJL074C*	*PTRG_01993*		*9292*	*211*	*4531*	*6204*	*AB08402*	X	
	SMC4	Subunit of the condensin complex; reorganizes chromosomes during cell division; forms a complex with Smc2p that has ATP-hydrolyzing and DNA-binding activity	*YLR086W*	*PTRG_03307*	*113706*	*5577*	*6106*	*10268*	*3889*	*AB01865*	X	
	SCC3	Necessary for sister-chromatid cohesion, required for double strand break repair	*YIL026C*	*PTRG_06072*	*32260*	*9620*	*2342*	*11181*	*986*	*AB04440*	X	
	PDS5	Essential for maintenance of sister chromatid cohesion	*YMR076C*	*PTRG_09677*	*97315*	*6088*	*9404*	*244*	*12335*	*AB06307*	X	